ISOLATION AND CHARACTERISATION OF *Crataeva magna* Lour (DC) ETHANOLIC EXTRACT AND ITS ANTI CANCER ACTIVITY AGAINST DALTON'S ASCITES LYMPHOMA CELL LINE

DISSERTATION

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The Tamilnadu Dr. M.G.R Medical University, Chennai

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K.M. COLLEGE OF PHARMACY

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TAMIL NADU

APRIL-2015

CERTIFICATE

This is to certify that the dissertation entitled **"ISOLATION AND CHARACTERISATION OF *Crataeva magna* Lour (DC) ETHANOLIC EXTRACT AND ITS ANTI CANCER ACTIVITY AGAINST DALTON'S ASCITES LYMPHOMA CELL LINE",** submitted by **Mr.K. NAGARAJAN,** in partial fulfilment for the degree of **"Master of Pharmacy in PHARMACEUTICAL CHEMISTRY"** under The Tamilnadu Dr. M.G.R Medical University, Chennai, at **K.M. College of Pharmacy**, Madurai, is a bonafide work carried out by him under my guidance and direct supervision during the academic year of **April 2014 – 2015**. This dissertation partially or fully has not been submitted for any other degree or diploma of this university.

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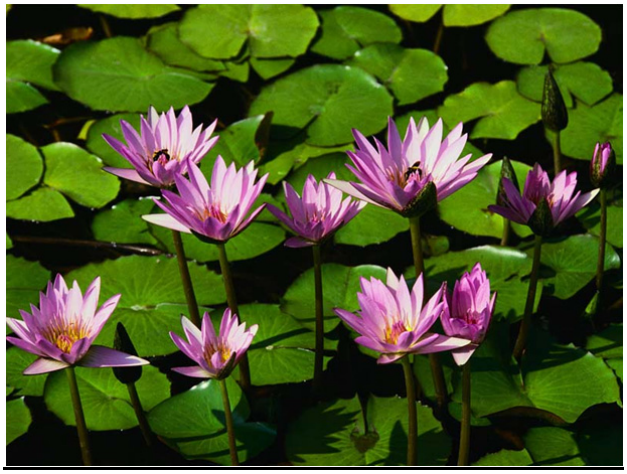
"Thank you All"

DEDICATED TO



THE ALMIGHTY AND MY FAMILY

CHAPTER I

INTRODUCTION

INTRODUCTION

"The emerging new technologies have significantly contributed in the advancements in developing new phytopharmaceuticals and food herbs, which are definitely going to alter the future outlook of family physicians and common people. India can play major role in the global market for herbals, herbal products, raw materials and isolated phytopharmaceuticals because of its extensive flora and fauna, expertise, trained technocrats and great plant heritage from Ayurveda and other resources**".**

Natural products including plants, animals and minerals have been the basis of treatment of human diseases and have been exploited for human use for thousands of years. The most important sources of drugs and drug leads in history, were introduced worldwide as drugs can be traced to or were inspired by natural products.[1] We were undergoing various works in identifying enormous natural resources which can be exploited to produce herbal medicines for the treatment of deadly ailments.

Natural products, the word itself begins with nature. Long back even before the evolution of modern science, there where diseases and there where cure too. At that age when people didn't know what is science and technology they used to cure diseases by the help of 'God's gift to the earth" that is his surroundings. What we see today is not the surrounding what they had at that time. It was pure nature at the beginning. The mixtures and blends what they made out of them cured the diseases. In modern world those treatment are named in different name like Ayurveda. Natural Products therapy and Homeopathy etc, it all derives from the same source, the source called Nature.

In the modern world the life and life style of human beings has changed rapidly. Now a day's the life of a normal human is almost sedentary. The major drawback of the sedentary lifestyle is obesity.[2] More than 20% of the world's population is obese. The major reason for this is due to the increased intake of Junk foods and oily foods that are supplied in fast-food corners. People are lazy to cook food at home. Another major problem what the modern world is facing is the Diabetics. Diabetes is almost everywhere, more than 50% of Indian population is diabetic. Even though these two diseases are there in the earth from a long time we humans are not able to find a permanent cure for both the problem. Today with the advanced scientific technology there have been a lot of inventions to counter this problem but nothing has worked out till this time.

Allopathic medicine had its birth in the 19th century during the American civil war. It was used as tools to give immediate results.[3] Hence it was termed as a wonder drug due to its immediate healing property. Meantime as per the ancient scripts people used to practice natural medicinal treatment from the first century only. The Chinese scripture shows the use of herbal medicines much before that. In olden days wounds where used to be healed by using a mixture of herbal pastes and herbal mixtures. The Indian herbal treatment method was known as Ayurveda, the European method was known as Homeopathy etc.

Today in our age as a result of constant natural exploitation to curb the hunger of Human greed we are in the verge of a mere natural disaster. What we see around us today is a concrete jungle instead of what it was years back. As the world developed the common health related problems became more common. These were not fully cured by scientific medicine and needed the gift of nature to be cured.

Today due to deforestation and natural exploitation the earth is in the brink of disaster. What we see around us today is a concrete forest built around us by humans. As the world developed the common heath related problems become more common which was not fully cured by scientific medicine and which needed the gift of nature to be cured. With the help of the old natural treatment scriptures, (Ayurveda, Chinese medicines etc) modern scientific world was able to rediscover their ancestors curing technique scientifically and what followed was an herbal revolution which we are seeing every day. "The specialty of herbal medicines is that, it was able to discover and develop the exact active component of a Natural medicine which helped in the curing of a disease". As a result of this invention today we are able to find a lot of natural products in the market in the form of Health Supplements in the form of food and beverage and various other products.[4]

The medicinal chemicals used throughout the world were isolated from natural sources. These sources include flowering plants, fungi, bacteria, and to a lesser extent, animals, especially marine animals. The subfield of organic chemistry that deals with isolating and studying chemicals found in nature is called natural products chemistry. Methods such as infrared and nuclear magnetic resonance spectroscopy, mass spectrometry, and X-ray methods are used to identify the structures of the compounds.[5] Laboratory synthesis of the compounds from simpler compounds provides confirmation of the structure

as well as a laboratory source of the chemicals. For further confirmation of structure, the isolated compound was chemically degraded to simpler compounds, which are more easily identified.

Traditional Medicines derived from medicinal plants are used by about 60% of the world's population. Diabetes is an important human ailment affecting many from various lives in different countries. In India it is proving to be a major health problem, especially in the urban areas. Though there are various approaches to reduce the ill effects of diabetes and its secondary complications, herbal formulations are preferred due to lesser side effects and low cost. A list of medicinal plants with proven anti-diabetic and related beneficial effects and of herbal drugs used in treatment of diabetes is compiled. One of the etiologic factors implicated in the development of diabetes and its complications is the damage induced by free radicals and hence an anti-diabetic compound with antioxidant properties would be more beneficial.[6]

Despite the ongoing research on synthetic compound, an equally concentrate and innovative interest in medicinal plants has ushered in. These natural medicinal substances are toxic potentially and produce certain amount of side effects, although treated with utmost care and caution. This has enhanced the instinct of all researchers, the world over, in formulating new Anti microbial agents and evaluating the efficacy or potentiality of natural products in substituting them as the alternatives for chemical (synthetic) antimicrobial agents.[7]

Herbal drugs are probably the most common source of samples for evaluation in high throughout screens of natural products. They have yielded many useful compounds and plant-derived ingredients, which are important components of modern phyto pharmaceuticals. Today, the global market is floated with herbal preparations. A number of companies, including some multinational are entering into the area of herbal medicines.[8-10]

Constituent of the component drug and reasonable mechanism of action to explain the therapeutic abilities, the elucidation of which must be a goal of oriental medicine research, plant whose constituents are isolated are used in allopathic medicine e.g. Atropine sulphate I.P.,Quinine sulphate I.P., Digoxin U.S.P.etc.

The disadvantage of oriental medicine is that the clinical use of drug is empirical and has been based on observation from clinical trials without experimental support. On the other hand as a great advantage, the efficacy has been already confirmed with humans. The development of science of phytopharmaceuticals in the western countries gave an impetus to the search for active principles in plants of Indian origin.

W.H.O GUIDELINES FOR ASSESSMENT OF HERBAL MEDICINES

Every herbal formulation must be standardized as per WHO guidelines. WHO collaborates and assists health ministries in establishing mechanisms for the introduction of traditional plant medicines into primary healthcare programmes, in assessing safety and efficacy and in ensuring adequate supplies and the quality control of raw and processed materials. HI according to WHO guidelines less stringent selection procedures could be applied for the screening, chemical analyses, clinical trials and regulatory measures but the procedure for pure phytochemicals for quality control should be identical to that for synthetic drugs according to WHO guidelines.

The World Health Organization (WHO) has recently defined traditional medicine as comprising therapeutic practices that have been in existence, often for hundreds of years, before the development and spread of modern medicine and are still in use today. The traditional preparations comprise medicinal plants, minerals, organic matter etc[11].

The manufacturing procedure and formula including the amount of excipients should be described in detail. The method of identification and quantification of the plant material in the finished product should be defined. If the identification of an active principle is not possible, it should be sufficient to identify a characteristic substance or mixture of substances (e.g. chromatographic fingerprint) to ensure consistent quality of the product. According to WHO, "Herbal Medicines" should be regarded as, "Finished, labeled medicinal products that contain active ingredients aerial or underground parts of plants, or other plant material, or combinations thereof, whether in the crude state or as plant preparations. Plant material includes juices, gums, fatty oils, essential oils, and any other substance of this nature. Herbal medicines may contain excipients in addition to the active ingredients. Medicines containing plant material combined with chemically defined active substances, including, isolated constituents of plants are considered to be herbal medicines. Exceptionally, in some countries

herbal medicines may also contain, by tradition, natural organic or inorganic active ingredients which are not of plant origin.

Multi-component botanical formulations can be standardized with newer techniques such as DNA fingerprinting, high pressure thin layer chromatography (HPTLC), liquid chromatography and mass spectroscopy. The value of animal testing to establish safety and toxicity is not so critical if the botanicals are used in traditional forms. Nevertheless all the critical pharmacopoeial tests such as dissolution time, microbial, pesticide and heavy metals contamination etc. must be in accordance with global standards and all the Ayurvedic medicine manufacture must be in accordance with current good manufacturing procedures for herbs[12].

HERBAL MEDICINE SCENARIO IN INDIA

The turnover of herbal medicines in India as over the counter products, ethical and classical formulations and have remedies of Ayurveda, Unani and Siddha systems of medicine is about $1 billion with a meager export of $ 80 million. 80% of the exports to developed countries are of crude drugs and not finished formulations leading to low revenue for the country. The list of medicinal plants exported from India are Aconitum species (root) Acorus *calamus* (rhizome), *Adatoda vasica* (whole plant), *Berberis aristata* (root), Cassia *augustifolia* (leaf and pod), *Colchicum luteum* (rhizome and seed), *Hedychium spicatum* (rhizome), *Heradeum candicans* (rhizome), *Inuia racemose* (rhizome), *Juglans reya* (husk), *Juniperus conimunis* (fruit), *Juniperus macropoda* (fruit), *Picrorhizn kurroon* (root), Plantago ovata (seed and husk), *Podophyllum emodi* (rhizome), *Pinicn. yanatum* (flower, root and bark), *Rauwolfia serpentina* (root). Rheum *emodi* (rhizome), Saussurea (rhizome), *Swertia shirayita* (whole plant), *Valerian- intamansi* (rhizome), Zingiber *officinale* (rhizome) Five of these, i.e. Glycerrhiza glabra, *Commiphora mukut,* Plantago *ovata. Aloe barbadensis* and *Azardica indica* are used in modern medicine. Others are used in 52 to 141 herbal formulations and Triphala (Terminalia *chebula,* and *Emblica officinalis]* along is used in 219 formulation[13].

India with its vast area from Kashmir to kanyakumari and varying soils and climatic conditions is a large store house of medicine plants, to be aptly called the "botanical garden of the world" and has a rich heritage of indigenous drugs from the Ayurvedic items.

The classical medicine system Ayurveda is strictly of Indian origin and development and it is still widely practised in India. More than 1500 remedial treatment with Indian medicine flora have been reported by Sushrata, Charaka and Vegbhatta in Sanskrit. The literature contain information about morphological features of many drugs, their geographical distribution and optimum condition for growth, the best season for their maximum potency as well as toxic properties thus a definite basis exists for investigating these plants for bioactive constituents[14].

The chemical analysis of crude drug helps us determining the action of medicine in health and disease. Today's emphasis of pharmaceutical research and development is on the search for the therapeutic substances with specific functions and minimum side effects in particular application. The plant derived substances having the advantages of being tools for medicine. Many different types of receptors were identified with the help of phytoconstituents e.g. muscarinic an active constituent of poisonous mushroom (amantia muscarrnia) was used to find both muscarinic & nicotinic receptors.

The natural plant product often serves as chemical models or templates for the design and total synthesis of new drug entities, e.g. new drug can be designed with the help of phytoconstituent, e.g. from morphine more than 25 synthetic congeners have been derived.

The wonder drugs of plant reported in recent years are ginsensosides from panax ginseng and have acquired commercial significance in view of its aphrodisiac and general tonic properties.

During the past decade investigation on secondary plants constituents have made phenomenal plant constituents have made phenomenal advances and thanks to the development of efficient separation techniques like column, thin layer, high pressure liquid and gas chromatography as well as sensitive methods of instrumental analysis such as UV, IR, NMR, ESR, ORD, CD and mass spectroscopy.

Modern instrumentation techniques have also made feasible, the study of micro quantities of substances with considerable precision in determining their chemical structure and distribution patterns in plants.

Recent reviews and book high light such investigation on the chemistry of medicinal plants will be more fruitful by close investigation with pharmacological and clinical investigation.

The technology involved in the extraction of pharmaceutical significance in majority of the cases is the guarded secret of the pharmaceutical or chemical firm. The method of extraction of phytopharmaceuticals represent the co-ordination of research work carried out by scientist different disciplines with the advancement in analytical and instrumentation technology, it has been possible to devise commercially feasible techniques for extraction of several phytochemicals[15].

The future of phytopharmaceuticals is bright as it undoubtedly serves as a cheep and steady for varied of therapeutic agents which are of great significance in the health care of mankind.

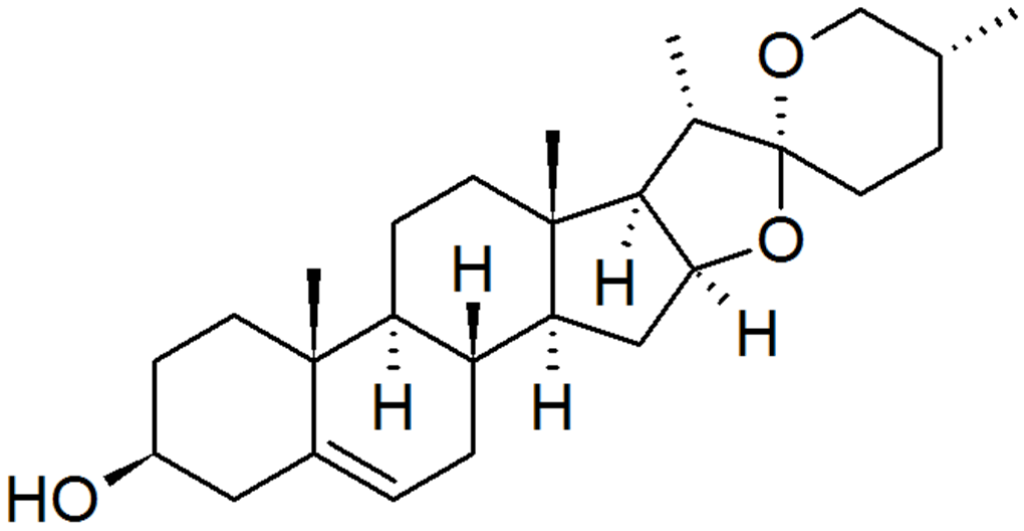
CHAPTER II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Mekap *et al.*,[16] investigated for its antiurolithiatic activity in two conventional models (*in vivo*) of Urolithiasis in rats. The two methods chosen were lactose (30%) + ethylene glycol (1%) and ammonium chloride (2%) + ethylene glycol (0.75%) induced urolithiasis, respectively. The ethanol extract (400 mg/kg bw) reduced the elevated levels of serum calcium (3.25 ± 0.30) and urine calcium (2.33 ± 0.18) significantly employing lactose (30%) + ethylene glycol (1%) induced urolithiasis model. The ethanol extract (400 mg/kg bw) reduced the urine uric acid level significantly employing both models, viz. lactose (30%) + ethylene glycol (1%) (0.82 ± 0.07; 0.001) and ammonium chloride (2%) + ethylene glycol (0.75%) (0.85 ± 0.12; 0.001) when compared to toxic group. The ethanol extract (400 mg/kg bw) employing both models resulted in reduced serum creatinine and calcium, urine oxalate and kidney weight significantly with a marked increase in final body weight and urine volume output when compared to toxic group. The results shown by the ethanol extract (400 mg/kg bw) group was compared to standard polyherbal drug (Cystone; 5 ml/kg bw) treated group and thus exhibited potent antiurolithiatic activity.

Amaresh Panda *et al.*,[17] designed to conduct phytochemical screening of *Crataeva magna* leaves after each successive extraction with petroleum ether, chloroform, methanol and water followed by its hepatoprotective activity study. Three different types of models used to examine the in vivo hepatoprotective activity of the above said extract were carbon tetrachloride, ethanol and paracetamol induced hepatotoxicity in rats and compared with silymarin (20 mg/kg) as reference standard. Phytochemical screening revealed the presence of carbohydrates, phenolic compounds, tannins, flavonoids, saponins and fixed oils in the aqueous extract of the *Crataeva magna* leaves. Two way analysis of variance study of the estimated biochemical parameters to illustrate, aspartate aminotransferase, alanine amino transferase and alkaline phosphatase were revealed that there is significant difference (p-value < 0.001) exists between the different treatment groups. Severe hepatic lesions induced by carbon tetrachloride, ethanol and paracetamol were significantly lowered after the administration of CM 200 mg/kg to the respective control groups (carbon tetrachloride > paracetamol > ethanol) which was also evident from the histopathological study of liver sections.



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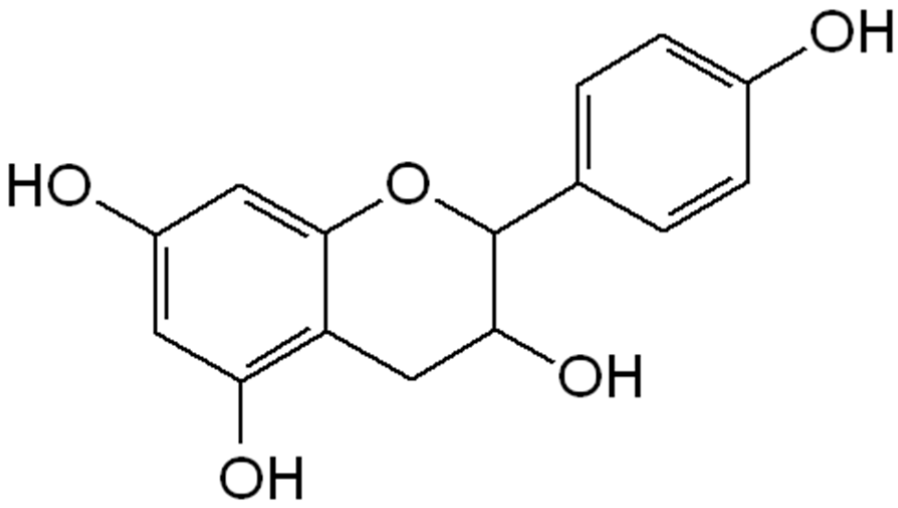

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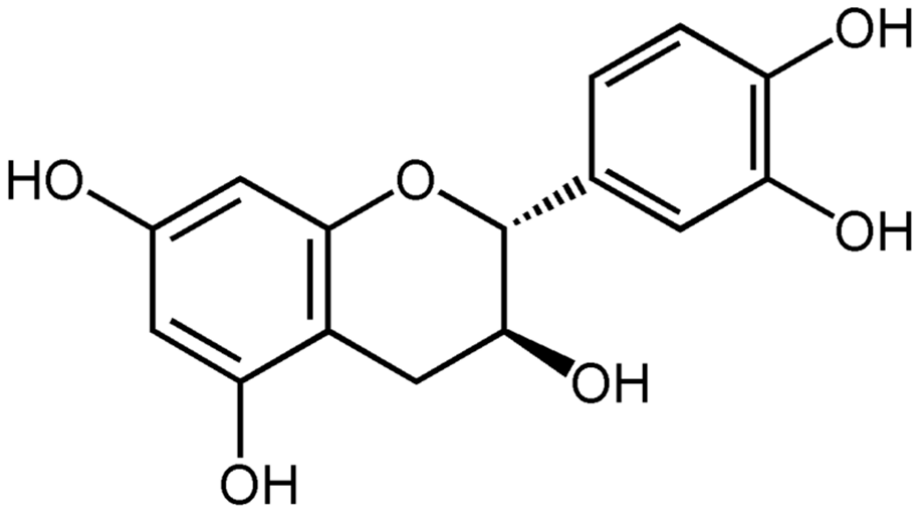

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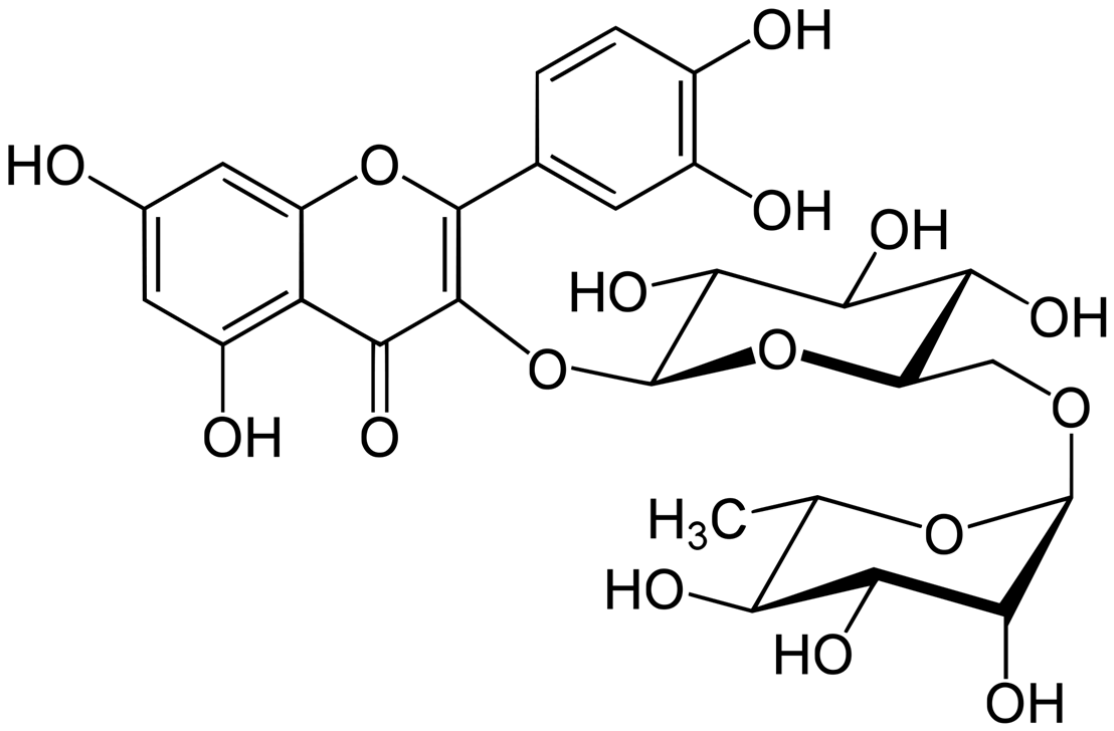

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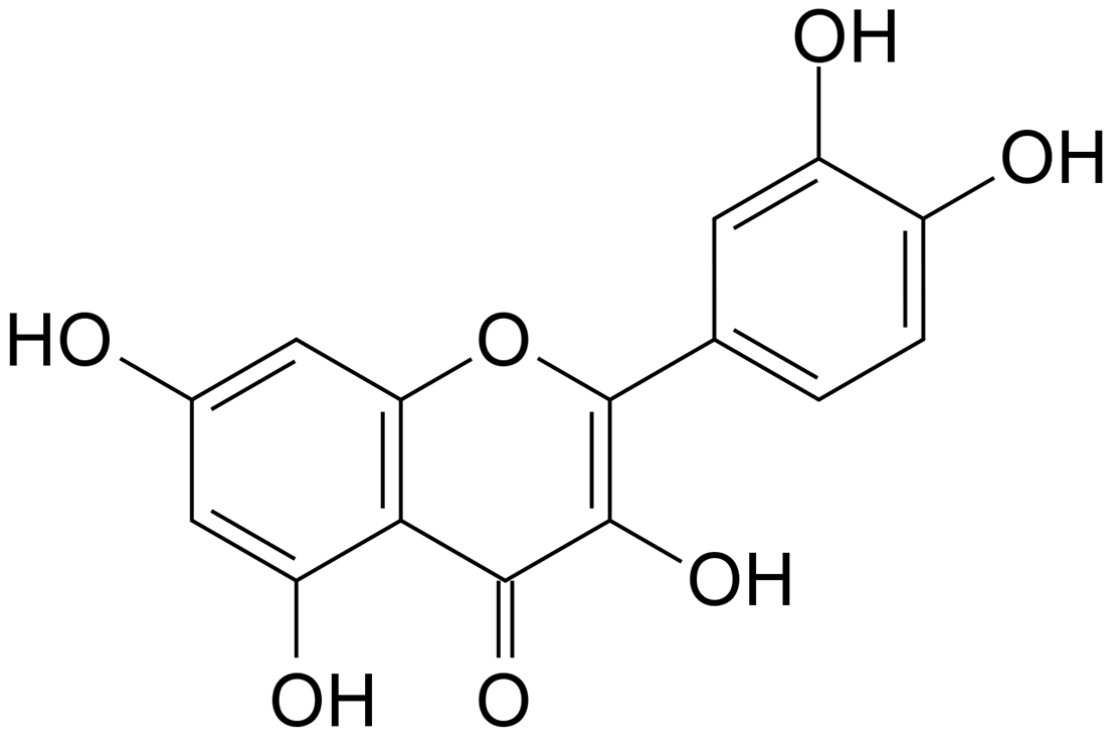

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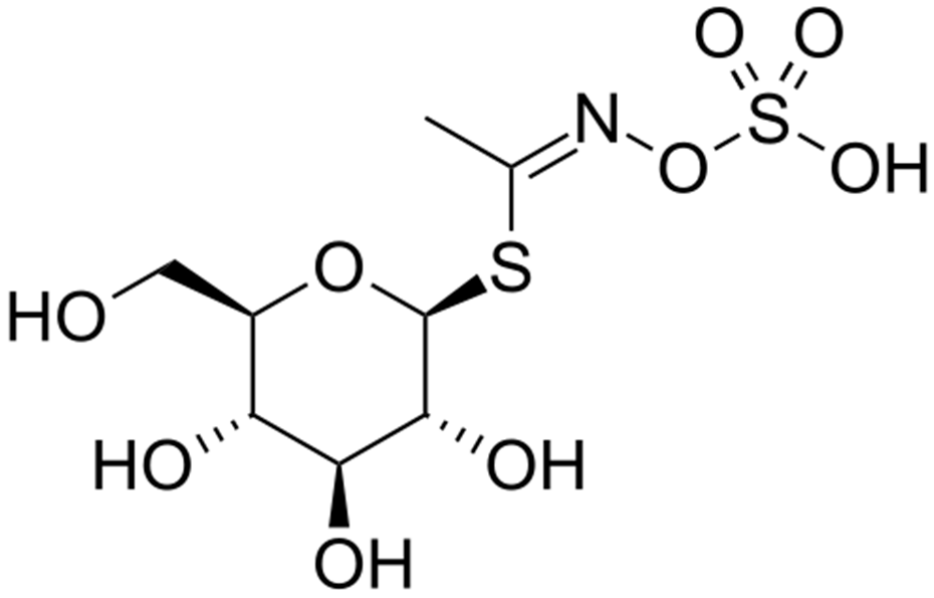

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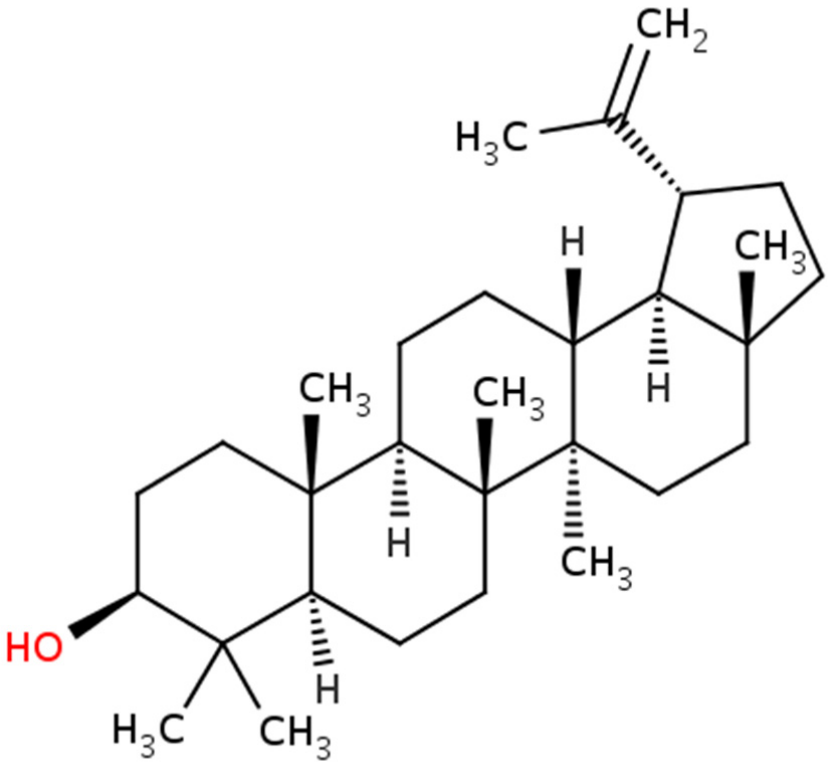

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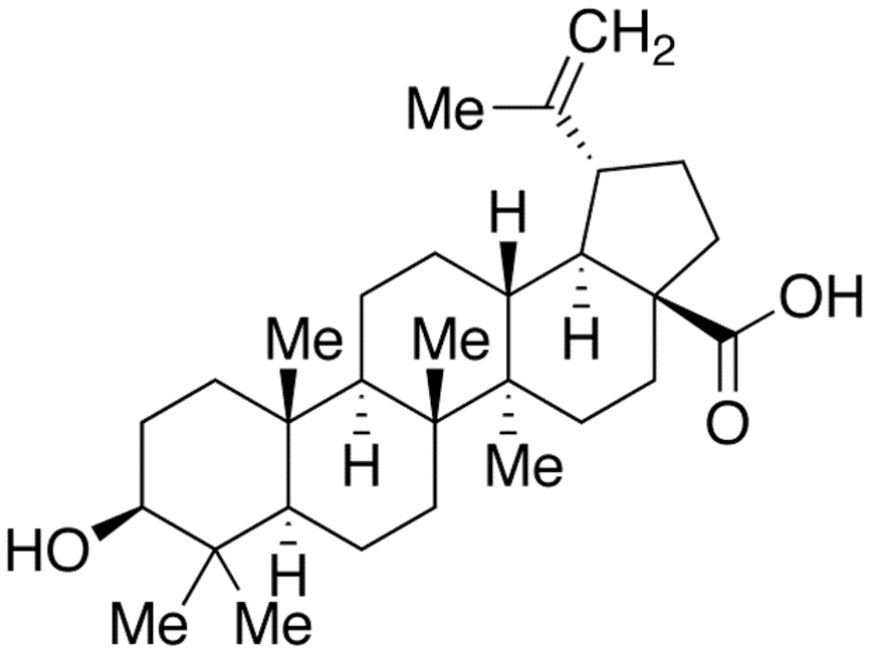
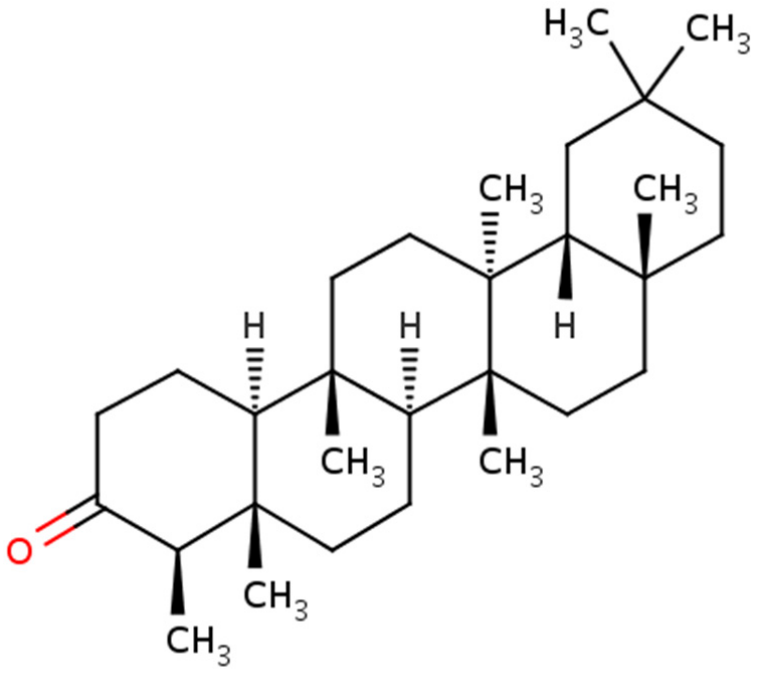


BETULINIC ACID



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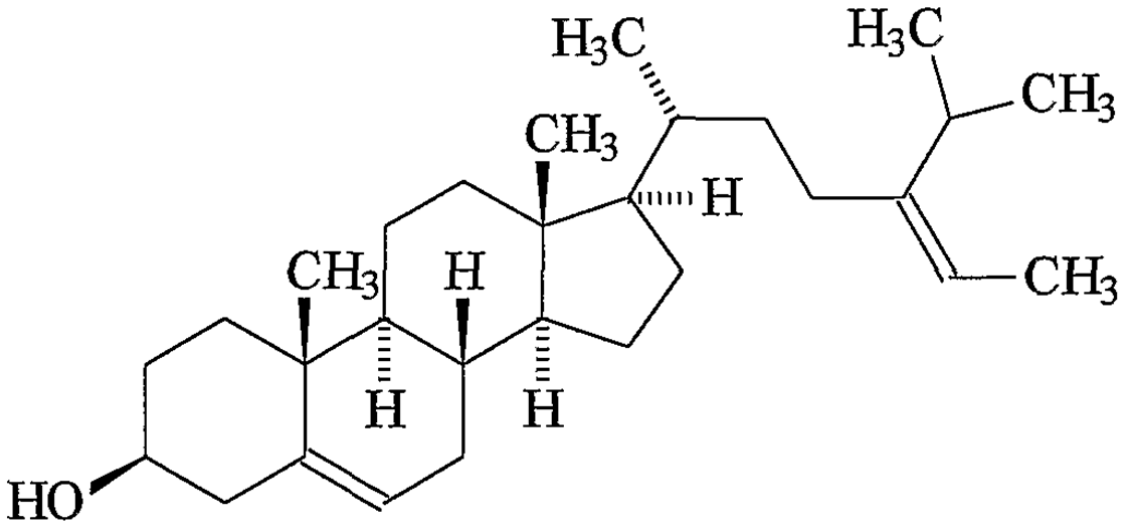


BETA- SETOSTEARY

Solomon Kiruba *et al.*,[18] studied it could serve as a pilot for the development of novel agents for various pathological disorders. However less information available regarding chemical constituents and bioactivity of this ethanomedicinally important species. Preliminary phytochemical screening of the pericarp of *Crataeva magna* proved the presence of phytochemicals such as phenols,saponins and tannins.

Monnanda Nalini *et al.,*[19] isolated Fungal endophytes from *Crataeva magna*, a medicinal plant growing along the streams and rivers, constituting riparian vegetation in Karnataka, southern India. Fresh bark and twig pieces were used for the isolation using standard methods. Ninety-six endophytic fungal isolates were isolated from 800 bark and twig segments. Mitosporic fungi represented as a major group (85%) followed by zygomycetes (10%) and ascomycetes (5%). Bark samples contained more endophytes than twig samples. Verticillium, Nigrospora oryzae and Fusarium verticilloides were the dominant fungal endophytes.

Bopana *et al.,*[20] developed of an efficient micropropagation protocol will play a significant role in meeting the requirement of quality planting material for commercial cultivation thereby conserving the species in its natural habitat. In the present study, shoot multiplication was achieved by culturing single node segments derived from a field grown tree on Murashige and Skoog's (MS) medium supplemented with 2.66 µM N6-benzyladenine, 1.39 µM Kinetin (Kn), 0.57 µM indole-3-acetic acid (IAA), 3% sucrose and 0.2% gelrite. 96% rooting was achieved within 22 days by culturing the in vitro formed shoots on half strength MS medium with 11.42 µM IAA, 9.8 µM indole-3-butyric acid, 0.46 µM Kn and 198.25 µM phloroglucinol. Following a simple hardening procedure involving sequential transfer of plants to a greenhouse, polyhouse, and shade net, the tissue-cultured plants were transferred to the field where the survival rate was 100%. To this date 500 plants have been produced. Inter simple sequence repeat analysis has confirmed genetic uniformity of the tissue-cultured plants.

Sovan Pattanaik *et al.,*[21] studied *Crataeva magna*, and Euphorbia nerifolia have been shown to possess hepatoprotective activity and antioxidant property. The plants were undertaken with the premise that the drug promoting hepatoprotective activity and radical scavenging property could have effect on wound healing also. The wound healing property of the methanolic extract of the leaves of *Crataeva magna* (CNM) and Euphorbia nerifolia

(ENM) were chosen to investigate in excision and incision wound models. The methanolic extracts of the two plants at the dose of 500 mg/kg/day by topically applying method. Healing was assessed by the rate of wound contraction, time until complete epithelialization, incision breaking strength, estimation of hydroxyproline and histopathological parameters. Complete wound contraction was shown by both the plants in the study period. In excision and incision wound models, all the test drugs showed significant ($P<0.001$) wound healing activities compared to the control. Moreover the CNM was found to possess significant wound healing activities than the ENM and had been observed to have equipotent wound healing activity as of the standard drug Framycetin.

Nataraj Loganayaki *et al.,*[22] investigated leaf and stem bark of *Crataeva magna* are evaluated for their antioxidant activity and inhibition of key enzymes relevant to hyperglycemia. Both the parts exhibited significant antioxidant and anti-α-glucosidase activity. The results will lead in favor of the use of this plant as a potential additive/nutraceutical antioxidant compound.

Gagandeep *et al.,*[23] investigated *Crataeva nurvala* leaves resulted in the isolation of four compounds, which are dodecanoic anhydride, methyl pentacosanoate, kaemferol-3-O-α-D-glucoside and quercitin-3-O-α-D-glucoside. Dodecanoic anhydride and methyl pentacosanoate are being reported for the first time from this plant. Kaemferol-3-O-α-D-glucoside and quercitin-3-O-α-D-glucoside have already been reported from this plant. Evaluation of free radical scavenging activity, wound healing activity and estimation of phenolic, flavonoid and proanthocyanidine contents of the plant "*Crataeva magna*".

Pattanaik *et al.,*[24] reported to prevent occurrence of many inflammatory and metabolic disorders. Pharmacological evaluation of *Crataeva magna* has revealed its potent activity against many diseases. This study evaluates the antioxidant capacity and wound healing activity of three extracts (petroleum ether,chloroform, methanol) were assessed against in vitro free radical scavenging activity by ABTS method, DPPH method and total ferric reductive potential and in vivo wound healing potential in rats respectively, suggesting its role as bioactive enhancer. The all three extract have antioxidant and wound healing activity, but the methanolic extract showed greater and pet ether extract showed the minimum free radical scavenging and wound healing activity. The methanolic extracts of the plant *Crataeva magna* posses higher phenolic, flavonoid and proanthocyanidine content than the other chloroform and pet ether extract.

Loganayaki *et al.*,[25] investigated leaf and stem bark of *Crataeva magna* are evaluated for their antioxidant activity and inhibition of key enzymes relevant to hyperglycemia. Both the parts exhibited significant antioxidant and anti-α-glucosidase activity. The results will lead in favor of the use of this plant as a potential additive/nutraceutical antioxidant compound.

Babu *et al.*, [26] evaluated the hepatoprotective activity of ethyl acetate extract of stem bark of *Crataeva magna* (cm) in CCl_4 induced toxicity in wistar albino rats. Stem bark of cm were collected, and subjected to continuous hot extraction in a soxhlet apparatus, for 72 h with solvents like chloroform, ethyl acetate and distilled water separately. Liver damage was induced in rats by administering CCl_4 subcutaneously (s.c) In the lower abdomen in a suspension of liquid paraffin (lp) in the ratio 1: 2 v/v at the dose of 1 ml CCl_4/kg b. Wt of each animal. CCl_4 was administered twice a week, on every first and fourth day of all 14 days. The extract at the dose of 200 mg/kg and 400 mg/kg b. Wt. Was evaluated by inducing hepatotoxicity with CCl_4 and using silymarin (100 mg/kg) as the reference standard. Biochemical parameters like, sgot, sgpt, salp and serum bilirubin level were analysed. The ethyl acetate extract of *Crataeva magna* possess significant hepatoprotection against CCl_4 induced hepatotoxicity in albino rats.

***Chadathorn Inyai et al.*,**[27] investigate new pancreatic lipase inhibitor from *Crataeva magna* (Kum-nam). The ethanolic extract of Kum-nam was screened for anti-lipase activity by using porcine pancreatic lipase assay. *p*-nitrophenylbutyrate was used as the substrate. It was found that, *Crataeva magna* extract showed an anti-lipase activity with the IC_{50} of 801.17µg/mL, while IC_{50} of Orlistat, positive control was 0.68µg/mL. The anti-lipase activity mechanism was mixed inhibition. Although, anti-lipase activity of Kum-nam was lower than Orlistat, further study of this plant should be conducted.

Jerajani *et al.*,[28] described that Purim tablet is a polyherbal formulation indicated for management of chronic dermatitis and it contains extracts of Curcuma longa, Cassia fistula, Psoralea corylifolia, Saussurea lappa, Picrorhiza kurroa, Azadirachta indica, Tinospora cordifolia, *Crataeva magna*, Eclipta alba, Andrographis paniculata, Emblica ribes, Emblica officinale, Terminalia chebula and Terminalia belerica. This study was planned to evaluate efficacy and safety of Purim tablet in chronic dermatitis. Aatopic dermatitis is an expression of immunologic malfunction, which manifests as a spectrum of complicated clinical

manifestations. This clinical trial observed highly significant rapid symptomatic relief and clinical improvement, with Purim tablets and there were no reported or observed adverse events. This study concluded that Purim tablets were clinically highly effective and safe in all types of chronic dermatitis.

Enamul Haque *et al.,*[29] evaluated that two triterpenoids, phragmalin triacetate (1**)** and lupeol (**2)** were isolated from an ethyl acetate extract of the stem bark of *Crataeva nurvala* (Capparidaceae) by repeated chromatography over silica gel. The structures of these compounds were determined by spectroscopic analyses (UV, IR, ^{1}HNMR, ^{13}C NMR and EIMS). This is the first report of the systematic phytochemical investigation and the presence of these compounds 1 and 2 from this plant.

Prabhat Das *et al.,*[30] evaluated the effect of *Crataeva magna*, whole plant (ethanolic and aqueous extract p.o.) on alloxan induced diabetes in appropriate animal model. The study was carried out on alloxan induced diabetic model. The diabetes was induced by using Alloxan and Glibencamide (5 mg/kg) was used as standard drug. The aqueous extract of leaves of *Crataeva magna*, results maximum yield value than that of petroleum ether extract, chloroform extract and alcohol extract through successive maceration process. The aqueous extract of leaves of *Crataeva magna* showed maximum control of blood sugar in hyperglycemic Wistar rats than other experimental extracts. The test extract also reduces the blood sugar level to a maximum extent in case of normal animals. So we finally came to the conclusion that the plant *Crataeva magna* increases healing of diabetes and prevents the development of experimentally induced diabetes in Wistar rats.

Sathya *et al.,*[31] standardized the physico-chemical traits of the Siddha polyherbal formulation Maavilingathy Mathirai (MLM). The organoleptic characters, physico-chemical characters like ash values, pH value, specific gravity, solubility were analyzed. The total ash value was found to be 9.2% w/w, acid insoluble ash value is 1.08% w/w, water soluble ash value is 2.9 %w/w, and loss of drying at 105ºC is 0.048 % w/w. The pH value is 7.4 and specific gravity is 0.925. The water soluble extractives and alcohol soluble extractives were found to be 7.93% w/w and 5.16 % w/w. The SEM analysis of the sample showed the presence of nano and micro particles. The quantitative analysis of the sample revealed the content of carbon, oxygen, magnesium, aluminium, silica, sulphur, chloride, potassium, calcium. The FT-IR spectroscopy applied in the mid infra-red region 4000 cm^{-1} to 400cm^{1} revealed the presence of functional groups like primary aliphatic amines, aromatics, alkanes,

alkynes, esters, ethers, alkyl halides. This study highlights the suitable application of modern standardizing techniques for bringing the herbal formulation into focus.

Atanu Bhattacharjee *et al.*,[32] *Crataeva nurvala* Buch-Hum (Varuna) is well known traditional Indian medicinal plant used to treat various ailments in particular urolithiasis. During last two decades, numerous ethno- pharmacological and scientific reports have been cited in the literature to support its multi- directional therapeutic potential. The plant is rich in alkaloids, saponins, triterpenes, tannins, flavanoid glycosides, glucosinolates and phytosterols. The review emphasizes primarily on folkloric uses, biological activities of isolated compounds, pharmacological activities of the extracts, clinical studies and safety profile of *Crataeva nurvala* to provide a comprehensive data for researchers to hit upon new chemical entity responsible for its claimed traditional uses.

Crataeva magna Lour.

CHAPTER III

AIM AND SCOPE OF PRESENT STUDY

AIM AND SCOPE OF PRESENT STUDY

In view of the various Phyto constituents and Pharmacological properties of the plant *Crataeva magna* Lour (DC), it was decided to work on the root bark which is used as indigenous medicine. The aim of the dissertation work was divided into the following areas

- Preparation of crude extracts by Hot percolation method using various solvents of increasing polarity.

- Preliminary screening of crude extracts obtained after solvent extraction and partial purification by Thin Layer Chromatography and Phyto chemical test analysis.

- Isolation and purification of selective phyto constituents by column chromatography.

- Characterization of the purified compounds by spectral methods.

- To study the pharmacological activities of the crude extracts.

CHAPTER IV

MORPHOLOGICAL STUDY OF *CRATAEVA MAGNA* LOURDC

MORPHOLOGICAL STUDY OF *Crataeva magna* Lour (DC)

LATIN BINOMIAL

Botanical Name : *Crataeva magna* Lour (DC)

Family : *Capparaceae*

VERNACULAR NAMES

Bengali	: Tikoshak
Gujarati	: Vayavarna
Hindi	: Barna
Kannada	: Narmbele,Varuna
Malayalam	: Nirvala
English	: Three lived caper
Tamil	: Maralingam
Telugu	: Uskia

ETHANOMEDICAL PROPERTIES AND USES

ROOT: The root of the plant have been used in Skin diseases, Astringent, Anthelmintic, Purgative, Emetic Diaphoretic, Antipyretic and Anti – tubercular properties. Root bark is an antidote for several poisons. Root bark is very bitter and is reputed as a cure for skin diseases[33].

STEM: The stem bark of the plant exerts a biphasic action and possesses marked hypotension in higher dose.

LEAF: The leaves of the plant was used as Anti - protozoa, Hypoglycemic, Spasmolytic**.** The leaves are applied as poultice in rheumatism.

FRUIT: Fruits are sweet, cooling, and purgative and used as a poultice for treating burning sensation and haemorrhage.

MORPHOLOGICAL CHARACTERS OF *Crataeva magna* Lour DC

A small tree growing upto10m height with wide spreading branches widely found in sub Himalayan tracts extending eastwards to Bihar, North Bengal, the hills of Assam, Meghalaya and Arunachal Pradesh and distributed throughout India[34].

Strong root yellow in colour with brown bark ; stem bark is pale brown coloured with shallow cracks, young parts pubescent ; leaves smooth, broad-ovate, entire to sub quadrate with large lobes ; flowers cream coloured or white in dense cymes; berries dark purple when ripe, ovoid; seeds slightly grooved, black on both faces with lateral white band.

The stem bark and leaves were collected at Tirunelveli District.

HABITAT AND DISTRIBUTION

The *Crataeva magna* Lour DC belongs to the family *Capparaceae* .A genus of shrubs or small trees, distributed in the Indo-Malaysian region, China, eastern Australia and tropical Africa. Four species occur in India[35].

A small tree, with spreading branches, found in the sub-Himalayan tracts and Kumaun, extending eastwards to Bihar, North Bengal, Hills of Assam, Meghalaya, Manipur and Arunachal Pradesh.

The tree occurs generally in moist ravines and is occasionally grown for hedges. It grows moderately fast. It is one of the recorded hosts of the Indian lac-insect in Assam. The leaves are lopped for fodder[36].

COLLECTION OF PLANT MATERIAL

The details regarding the description and identification of plant *Crataeva magna* Lour DC were already given. The plant *Crataeva magna* Lour DC was collected from Tirunelveli during the month of May. Then was identified by *V.Chelladurai (Reasearch officer, Department of Botany, Central council for research in ayurvedha and siddha, Government of*

*India).*The root bark were dried in shade for 45 days. Then about 3 kg of the shade root bark was made in to coarse granules and was used for different investigation[37, 38].

CHAPTER V

PHYTOCHEMICAL INVESTIGATION OF THE ROOT EXTRACT OF *CRATAEVA MAGNA* LOUR DC

PHYTOCHEMICAL INVESTIGATION OF THE ROOT EXTRACT OF

Crataeva magna Lour (DC)

Preparationof root bark extract using different solvents of increasing Polarity. Screening for the detection of various plant constituent on the following

SOLVENT EXTRACTION (HOT PERCOLATION METHOD)

Material used where R.B flask condenser, Petroleum ether AR, Ethanol AR and shade dried root bark of *Crataeva magna* Lour DC[39].

METHOD

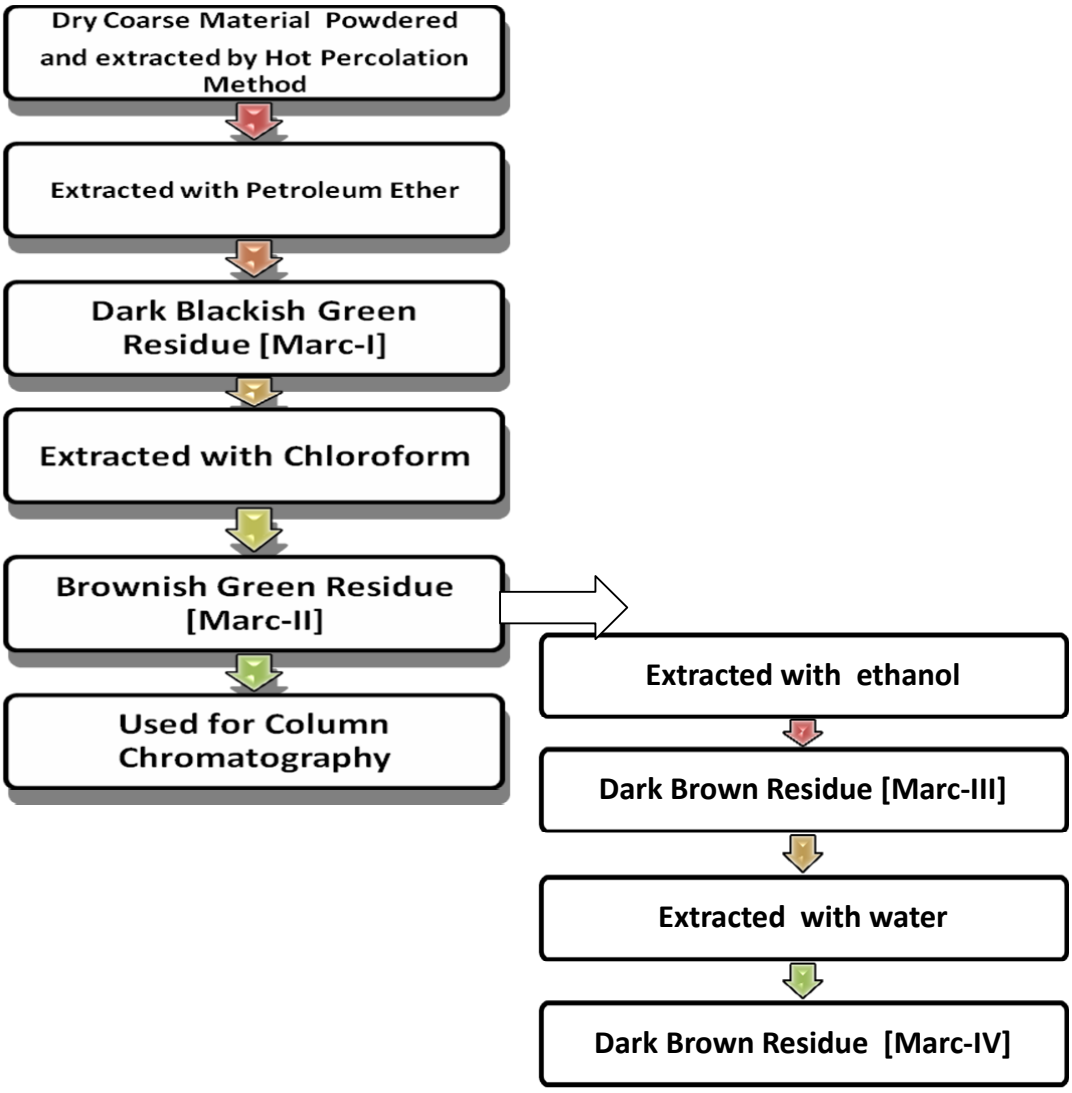
The root bark of *Crataeva magna* Lour DC where dried in the shade then the shade dried root bark where powdered to get a coarse powder. About 350 gms of dried course granules of *Crataeva magna Lour* DC root bark was soaked into 4 litres. Petroleum ether for 2 days, then it was extract first with petroleum ether at 40 – 60ºC by continuous hot percolation method using R.B flask apparatus. The extraction was continued for 72 hours. The petroleum ether extract was filtered and concentrated to a dry mass by distillation (16 gm) a dark blackish green residue was obtained. The marc left, after petroleum ether extraction was taken and then subsequently extract with Chloroform and ethanol for 72 hours. The ethanolic extract was taken and the filtered and concentrated to the dry mass. A dark brown residue was obtained (20 gms).

The extract above obtained named as

- Petroleum ether extract
- Chloroform extract
- Ethanol extract

FLOW CHART OF VARIOUS EXTRACTION AND ISOLATION OF COMPOUNDS *Crataeva magna* Lour DC

SCHEMATIC DIAGRAM:1



CHAPTER VI

QUALITATIVE CHEMICAL EVALUATION

QUALITATIVE CHEMICAL EVALUATION[40-42]

The extracts obtained by the above methods were subjected to qualitative test for the identification of various plant constituents.

DETECTION OF CARBOHYDRATES

Small quantities of different extracts were dissolved separately in 5 ml of distilled water and filtered. The filtrate was subjected to Molish's test and Fehling's test to detect the presence of carbohydrates.

a) Molish's test:

To the filtrate 2-3 drops of 1% alcoholic alpha naphthol and 2 ml of concentrated sulphuric acid was added along the sides of test tube. A purple colour ring was formed at the junction of the two liquids in Ethanol, chloroform, Aqueous extracts. No purple ring was formed in Pet ether extract. It showed negative response for Pet ether extracts for the presence of carbohydrates.

b) Fehling's test :

Small portion of various extracts were treated with Fehling's solution 1 and 2 and then heated. A red precipitate was seen in chloroform, Aqueous extracts, Ethanol extracts. No red precipitate was seen in Pet ether extract.

DETECTION OF GLYCOSIDES

Small quantity of various extracts were hydrolyzed with hydrochloric acid for two hours on a water bath and the hydrolysate was subjected to

a) Legal's and b) Borntrager's test to detect the presence of different glycosides.

a) Legal's test:

To the hydrolysate extract, 1 ml of pyridine and few drops of sodium nitroprusside solution were added then it was made alkaline with sodium hydroxide solution. Appearance of pink to yellow colour was observed, it showed the presence of glycosides in all the extracts.

b) Borntrager's test:

Hydrlosate extract was treated with chloroform and chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. Appearance of pink colour was observed in ammoniacal layer in all the extracts.

c) Modified Borntrager's test:

The extracts were boiled with few ml of dilute hydrochloric acid and 5 ml of ferric chloride solution. The contents are cooled and shaken with organic solvents. Organic layer was separated and to this equal volume of ammoniacal solution was added. The ammoniacal layer showed pink colour in all the extracts. In this test, addition of ferric chloride was added to break the C-C linking of glycosides which is stronger than C=O linkage.

DETECTION OF ALKALOIDS

Small quantity of extracts were separately treated with a few drops of dilute hydrochloric acid and filtered. The filtrate was treated with various alkaloids reagents such as a) Wagner's reagent b) Dragendroff's reagent c) Mayer's reagent and d) Hager's reagent.

a) Wagner's reagent:

To 1 ml of the filtrate few drops of Wagner's reagent, was added. Reddish brown precipitate produced in all the four extracts, indicating the presence of alkaloids.

b) Dragendroff's test:

To 1 ml the filtrate few drops of Dragendroff's reagent was added Orange red precipitate produced in all the four extracts, indicating the presence of alkaloids.

c) Mayer's test:

To 1 ml of the filtrate few drops of Mayer's reagent was added. Cream precipitate produced in all the four extracts, indicating the presence of alkaloids.

d) Hager's test:

To 1 ml of the filtrate few drops of Hager's reagent was added.Yellow precipitate produced in both Petroleum ether and ethanol extracts indicating the presence of alkaloids. No Yellow precipitate produced in Chloroform and aqueous extracts.

DETECTION OF PHYTOSTEROLS

Small quantity of various extracts was dissolved in 5 ml of chloroform separately. Then these chloroform solutions were subjected to a) Salkowski test b) Libermann - Burchard's test, for the detection of phytosterols.

a) Salkowski test:

To 1 ml of the above prepared chloroform solution, few drops of concentrated sulphuric acid was added and allowed to stand after shaking. chloroform and ethanol extract produced red colour in the lower layer. It showed the presence of phytosterols.

b) Libermann – Burchard test:

The above chloroform solution was treated with few drops of acetic anhydride and 1ml of concentrated sulphuric acid along the sides of the test tube and allowed to stand. Green colour was produced chloroform and ethanolic extract indicating the presence of phytosterols.

DETECTION OF SAPONINS

Small quantities of extracts were diluted with 20 ml of distilled water and it was agitated in a graduated cylinder for 15 minutes. A 1 cm layer of foam was produced in all the four extracts indicating the presence of saponins.

DETECTION OF FIXED OILS AND FATS

a. Small quantity of various extracts was separated between two filter papers. Oil stains were not produced, which showed the absence of fixed oils.
b. Few drops of 0.5 N alcoholic potassium hydroxide was added to various extracts with a few drops of phenolphthalein. The mixture was heated on a water bath for about 2 hours. Soap formation was seen in chloroform, ethanol and Pet ether extracts

DETECTION OF TANNINS

All the extracts were dissolved separately in minimum amount of water and filtered.

a) Gelatin test:

To the filtrate added 1 ml of 1% solution of gelatin. White precipitate was observed in Pet ether; chloroform Extracts indicating the presence of tannins.

b) **Ferric chloride test**:

To the filtrate few drops of ferric chloride solution were added. Bluish black precipitate was produced in Pet ether and Chloroform extracts indicating the presence of tannins.

DETECTION OF PROTEINS AND AMINOACIDS

Small quantities of the different extracts were dissolved in few ml of water and they were subjected to a) Millon's test b) Biuret test c) Ninhydrin test.

a) Millon's Test :

The above extracts were treated with Millon's reagent. Pet ether and chloroform extracts produced white precipitate which turns red on heating.

b) **Biuret Test** :

The above extracts were warmed gently with equal volume of 10% sodium hydroxide and few drops of 1% copper sulphate were added.Reddish violet colour was produced in Pet Ether and chloroform extracts .

C) Ninhydrin Test:

The above extracts were treated with Ninhydrin reagent. No blue or pink colour was produced in Aqueous and alcohol extracts. Pet ether and chloroform extract produced blue colour indicates the presence of proteins and amino acids respectively.

DETECTION OF FLAVONOIDS

The different extracts were separately dissolve in alcohol solution and then subjected to the following test.

a) Shinoda's Test:

To the alcoholic solution a small piece of magnesium metal followed by concentrated hydrochloric acid was added drop wise and heated. Magenta colour was produced in Ethanol, chloroform and aqueous extracts indicating the presence of flavonoids.

b) Ferric chloride Test

To the alcoholic solution few drops of neutral Ferric chloride solution was added. Blackish red precipitate was observed in Ethanol, chloroform and aqueous extracts indicating the presence of flavonoids.

DETECTION OF FLAVONES

a) With sodium hydroxide solutions the extracts gave yellow colour.

b) With concentrated sulphuric acid orange colour was produced.

c) ZINC, HYDROCHLORIC ACID REDUCTION TEST

To a small quantity of each extracts a pinch of Zinc dust was added. Then added a few drops Con.Hydrochloric acid. Magenta colour was produced.

d) LEAD ACETATE SOLUTION TEST

To a small quantity of each extracts a few drops of 10% lead acetate solution was added Yellow precipitate was produced indicating the presence of flavones in all the extracts.

DETECTION OF COUMARINS

a) The small quantity of extracts were dissolved in alcohol and exposed to U.V light. It produce green colour.

b) All the extracts were dissolved in alcohol and added with few drops of Ferric chloride solution green colour was obtained.

All the extracts showed the presence of coumarins.

DATA SHOWING THE PRELIMINARY PHYTOCHEMICAL SCREENING OF THE VARIOUS EXTRACTS OF *Crataeva magna* Lour DC

TABLE NO. 1

Consituents	Pet ether extract	Chloroform extract	Ethanol extract
Carbohydrates	-	+	+
Glycosides	+	+	+
Alkaloids	+	+	+
Phytosterols	-	+	+
Saponins	+	+	+
Fixed oils & fat	+	+	+
Tannins	+	+	-
Protein & Amino Acids	+	+	-
Flavonoids	-	+	+
Coumarins	+	+	+
Flavones	+	+	+

\+ - Indicate positive test results

\- - Indicate negative test results

These crude extract were also used for the exhibition of some selective pharmacological properties.

BEHAVIOUR OF POWDER

Coarsely powdered root bark of *Crataeva magna Lour DC* was treated with various reagents and observed and shown in the Table No.2.

TABLE NO.2

REAGENTS	COLOUR/PPT	CONSTITUENTS
Powder as such	Brownish Green	-
Powder+ conc. Sulphuric acid	Brown colour	Steroids present
Powder+Aqs Ferric Chloride	No Black colour	Tannins absent
Powder+ Iodine Sol.	No Blue colour	No Starch Present
Powder+ Aqs. Mercuric Chloride	Brown colour	Alkaloids Present
Powder+Picric Acid	Slight Yellow colour	Alkaloids Present
Powder+Mg.HCL	Light Yellow colour	Flavonoids Present
Powder+ Ammonia	Reddish Brown	Anthraquinone Present
Powder + Aqs.KOH	No Change	Anthraquinone Present

CHAPTER VII

ISOLATION AND PURIFICATION OF ISOLATED COMPOUNDS

ISOLATION, PURIFICATION AND IDENTIFICATION OF THE CONSTITUENTS OF *Crataeva magna* Lour DC

COLUMN CHROMATOGRAPHY OF ETHANOLIC EXTRACT[43]

10 gm of crude ethanolic extract was packed with 100 gms of silica gel (60-120) mesh using 2.4 diameter column was packed with mixture of ethyl acetate and hexane. The column was eluted with increasing solvent polarity from hexane to ethyl acetate.

PREPARATION OF COLUMN

The glass column of 3 cm diameter was packed with activated silica gel (100 – 120 mesh) slurry in petroleum ether to a height of 45 cm in order to establish a column of 300 ml. The column was developed according to the following lines. The column was built up by passing one-column volume of petroleum ether before the residue was loaded. The solvent was kept 5 cm above the bed and the residue was carefully loaded in the form of petroleum ether slurry. The column was then developed with a series of solvent starting with Petroleum Ether, Hexane, Ethyl Acetate and Methanol in increasing polarity.

The different ratios with succeeding solvents were fixed and are shown in the table no 2. Fractions of 100 ml were collected up to methanol system and thereafter, fraction in smaller volumes are collected checked with TLC and concentrated and processed further.

PREPARATION OF THIN LAYER CHROMATOGRAPHY PLATES[44]

About 50 gm silica gel G was weighed and it was shaken with 100 ml water to form a homogenous suspension. This suspension was poured into a thin layer chromatography applicator, which was adjusted to 0.25 mm thickness.

The plates were kept in hot air oven at 110 °C for 1 hour to activate the silica gel G. They were then stored in dry atmosphere and used when ever required.

By using the capillary tube, the elutes were spotted on the TLC plates around 2 cm above its bottom and subjected to chromatogram with different solvent systems. The compounds moved according to their affinity towards different solvent system.

The plates after development in each solvent system were observed under UV lamp. The different spots developed in each solvent systems were identified and by means of different spraying systems and also by placing in iodine chamber. The compounds moved relative to their affinity.

$$R_f = \frac{\textbf{Distance travelled by solute}}{\textbf{Distance travelled by solvent front}}$$

DATA SHOWING VARIOUS CONSTITUENTS PRESENT IN THE EXTRACTS BY THIN LAYER CHROMATOGRAPHY

TABLE NO.3

S.No	Active Constituents	Mobile Phase	Spraying Reagent	Inference
1	Alkaloids	Methanol : Ammonium Hydroxide 5 : 5	Dragendorff reagent	Orange Brown R_f=0.41
2	Cardiac Glycosides	Ethyl Acetate : Hexane 3 : 7	Anisaldehyde sulphuric Acid	Orange Brown R_f =0.6;0.7
3	Flavonoids	Butanol : Acetic Acid :Water : Ether 9 : 6 : 1 : 3	Phenol Sulphuric Acid	Greenish Brown R_f =0.46;0.52
4	Phytosterols	Hexane : Diethyl Ether 32 : 1	Stannic Chloride	Orange Brown R_f =0.6;0.54
5	Carbohydrates	Butanol : Acetic Acid :Water : Ether 9 : 6 : 1 : 3	Phenol Sulphuric Acid	Greenish Brown R_f=0.52;0.63
6	Tannins	Toluene : Acetone 9 : 1	Ferric Chloride	Black observed R_f=0.8;0.6

DATA SHOWING THE COLUMN CHROMATOGRAPHY OF ETHANOLIC EXTRACT

TABLE NO.4

S.No	Number of fractions	% of solvent	Volume of solvent (ml)	Compounds
1	1-50	98%Hex:2%Etoac	600	
2	51-90	95%Hex:5%Etoac	500	
3	91-120	90%Hex:10%Etoac	500	
4	121-160	85%Hex:15%Etoac	600	
5	161-180	80%Hex:20%Etoac	800	Compound 1
6	181-200	75%Hex:25%Etoac	800	
7	201-220	100%Etoac	400	
8	221-238	98%Etoac:2%MeoH	350	Compound 2
9	239-250	90%Etoac:10%MeoH	250	

Etoac –Ethylacetate, MeoH- Methanol

TLC PROTOCOL

TABLE NO.5

FRACTIONS	ELUTES SYSTEM	R_f VALUES
CM I	BENZENE: CHLOROFORM (8:2)	0.42
CM II	CHLOROFORM: ETHYL ACETATE(7:3)	0.71

CHAPTER VIII

IDENTIFICATION OF ISOLATED COMPOUND

IDENTIFICATION OF ISOLATED COMPOUNDS[45-47]

COMPOUND CM– I

The compound was isolated from the dried root bark of *Crataeva magna* Lour DC

Appearance : Dark Green residue

Yield Obtained : 120 mg

Solubility : Soluble in Benzene and chloroform

Melting point : 142 ° C

CHEMICAL TEST

TEST FOR ALKALOIDS

1. **Mayer's Test**

A small quantity of compound CM-I was mixed with dil. HCL and to this one-drop of Mayer's reagent (Postassium mercuric iodide) was added. It produced a precipitate, indicating the presence of alkaloids.

2. **Dragendorff's Test**

A small quantity of compound CM-I was mixed with dilute HCL, followed by 1 ml of dragendorff's reagent (Postassium bismuth iodide). An orange red precipitate was formed.

3. **Wagner's Test**

A small quantity of compound CM-I was mixed with 1 ml of dil. HCL followed by 1ml of Wagner's reagent (Iodide solution) a brown precipitate was produced.

4. **Hager's Test**

A small quantity of compound CM-1 was mixed with 1ml of dil. HCL, follwed by 1 ml of Hager's reagent (Picric acid) a yellow precipitate was produced.

TLC SYSTEM:

Benzene : Chloroform (8:2)

R_f : 0.42

Spraying Reagent : Dragendorff Reagent, Iodine chamber

IR SPECTRUM OF COMPOUND CM-1

IR Spectrum was taken using neat on Perkin Elmer model IR spectrophotometer, KBr-media. The spectrum was attached. The IR peak and the groups assigned are shown in Table No:6

NMR SPECTRA OF COMPOUND CM-1

NMR was taken on Avance 300, ^{1}HNMR ($CDCl_3$) 300 MHz, TMS as standard as shown in the Table No. 7 and the spectrum were attached.

TABLE NO.6

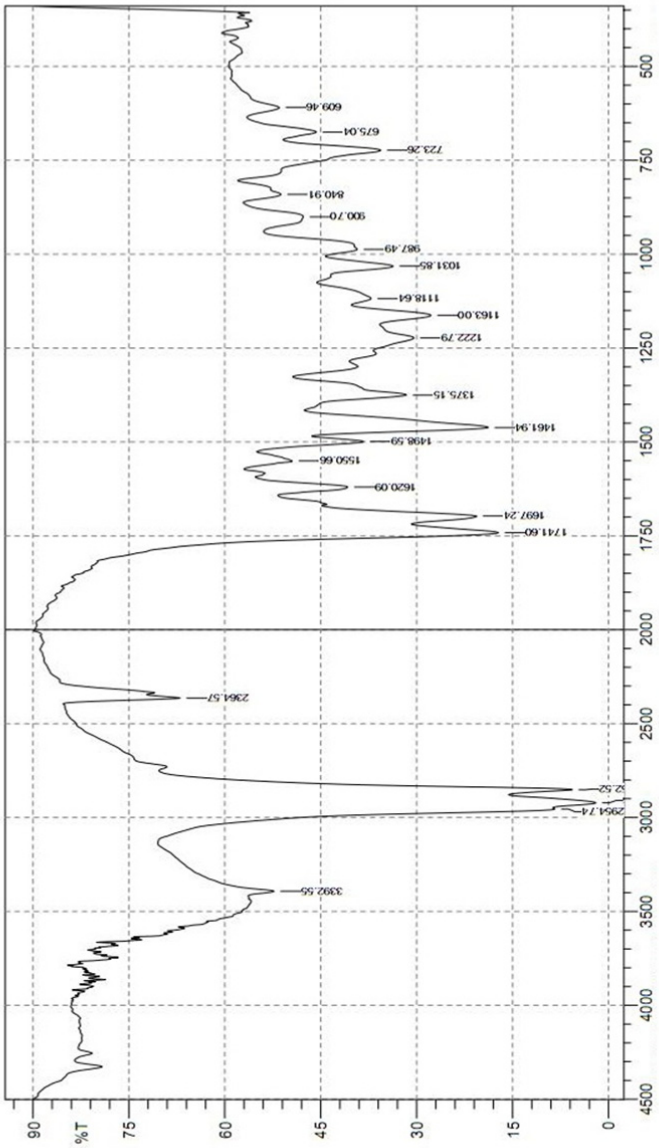
COMPOUND CM I

S.No	Frequency (cm^{-1})	Groups Assigned
1	3392.55	May be due to O-H stretching
2	2364.57	May be due to C-H stretching
3	1741.60	May be due to C=O stretching
4	1697.24	May be due to C=O stretching
5	1498.59	May be due to C-H bending
6	1461.94	May be due to C-H bending
7	1222.79	May be due to C-O stretching
8	1163	May be due to C-O stretching
9	723.26	May be due to C-H bending

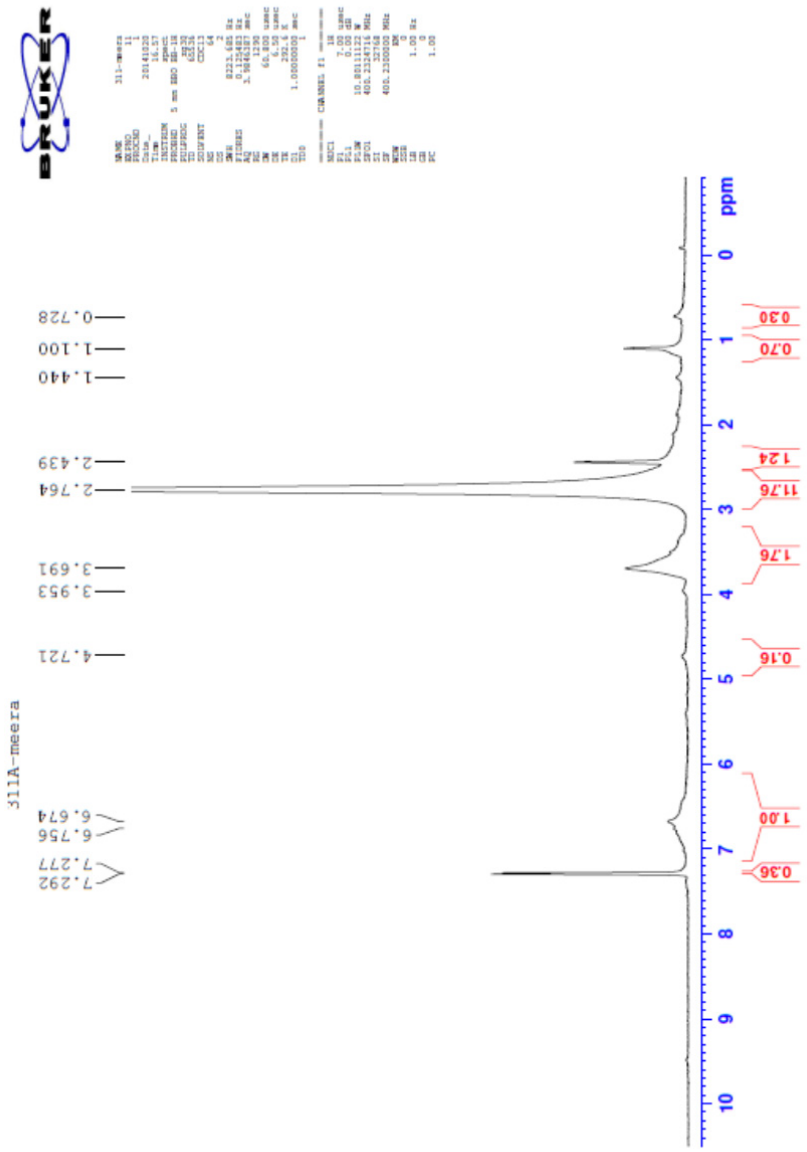
TABLE NO.7

S.No	SIGNALS (δ) VALUES ppm	GROUPS ASSIGNED
1	0.745	Due to CH_3 protons
2	0.929	Due to CH_3 protons
3	1.034	Due to CH_3 protons
4	1.137-1.515	Due to CH_3 protons
5	1.807-1.957	Due to CH protons
6	2.13	Due to CH_2 proton attached near to CH_2-C=O
7	2.462	Due to CH_2 proton attached near to CH_2-C=O

IR SPECTRUM OF COMPOUND CM -I



NMR SPECTRUM OF COMPOUND CM-I



COMPOUND CM – II

The compound was isolated from the dried root bark of *Crataeva magna* Lour DC.

Appearance	:	Green
Yield Obtained	:	90 mg
Solubility	:	Soluble in Benzene and chloroform
Melting point	:	138°C

CHEMICAL TEST

TEST FOR GLYCOSIDES

Small quantity of the compound CM II were hydrolyzed with hydrochloric acid for two hours on a water bath and the hydrolysate was subjected to

a) Legal's b) Borntrager's and c) Modified Borntrager's test to detect the presence of different glycosides.

a) Legal's Test:

To the hydrolysate, 1 ml of pyridine and few drops of sodium nitroprusside solution were added then it was made alkaline with sodium hydroxide solution. Appearance of pink to yellow colour was observed; it showed the presence of glycosides

b) Borntrager's Test:

Hydrlosate was treated with chloroform and chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. Appearance of pink colour was observed in ammoniacal layer.

c) Modified Borntrager's Test:

The hydroslate compound was boiled with few ml of dilute hydrochloric acid and 5 ml of ferric chloride solution. The contents are cooled and shaken with organic solvents. Organic layer was separated and to this equal volume of ammoniacal solution was added.

The ammoniacal layer showed pink colour. In this test, addition of ferric chloride was added to break the C-C linking of glycosides which is stronger than C=O linkage.

TLC SYSTEM:

Chloroform : Ethyl Acetate (7:3)

R_f : 0.71

IR SPECTRUM OF COMPOUND CM-II

IR Spectrum was taken using neat on Perkin Elmer model IR spectrophotometer (KBr) Media. The spectrum was attached. The IR peak and the groups assigned are shown in Table No:8

NMR SPECTRA OF COMPOUND CM-II

NMR was taken on Avance 300, ^{1}HNMR ($CDCl_3$) 300 MHz, TMS as standard as shown in the Table No.9 and the spectrum were attached.

TABLE NO. 8

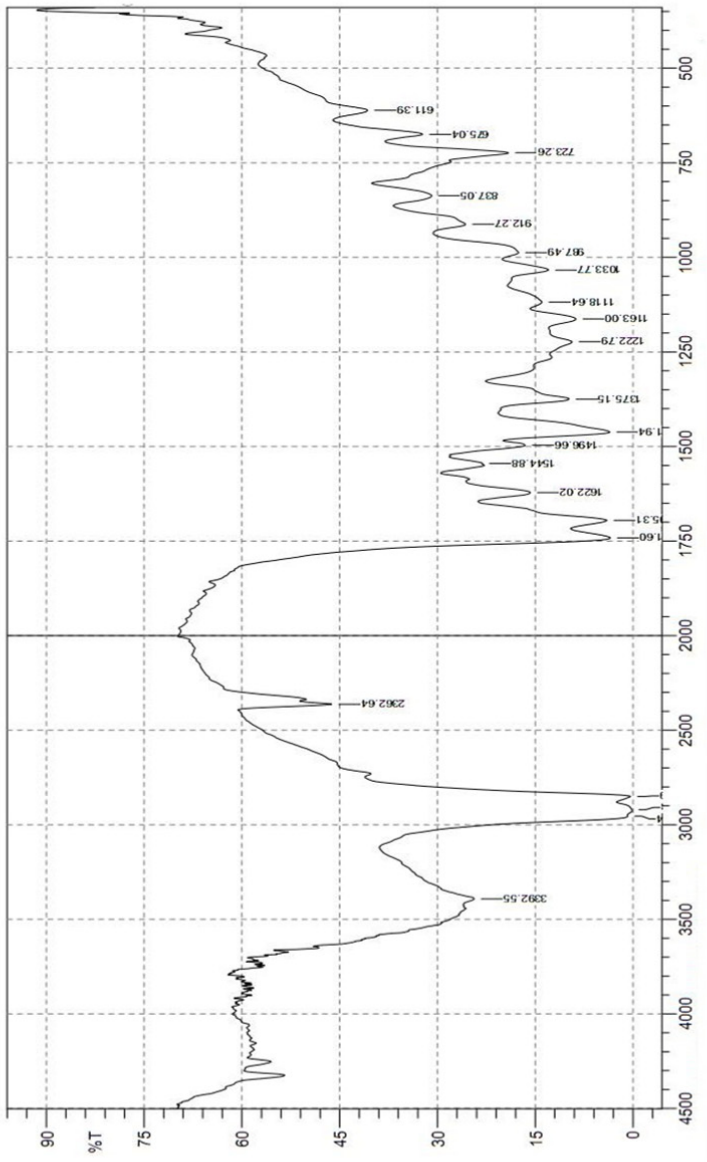
Compound - CM II

S.No	Frequency (cm^{-1})	Groups Assigned
1	3392.55	May be due to O-H stretching
2	2362.64	May be due to C-H stretching
3	1622.02	May be due to C=O stretching
4	1544.88	May be due to N-H bending
5	1375.15	May be due to C-H bending
6	1222.79	May be due to C-O stretching
7	1163	May be due to C-O stretching
8	1033.77	May be due to C-O stretching
9	723.26	May be due to C-H bending

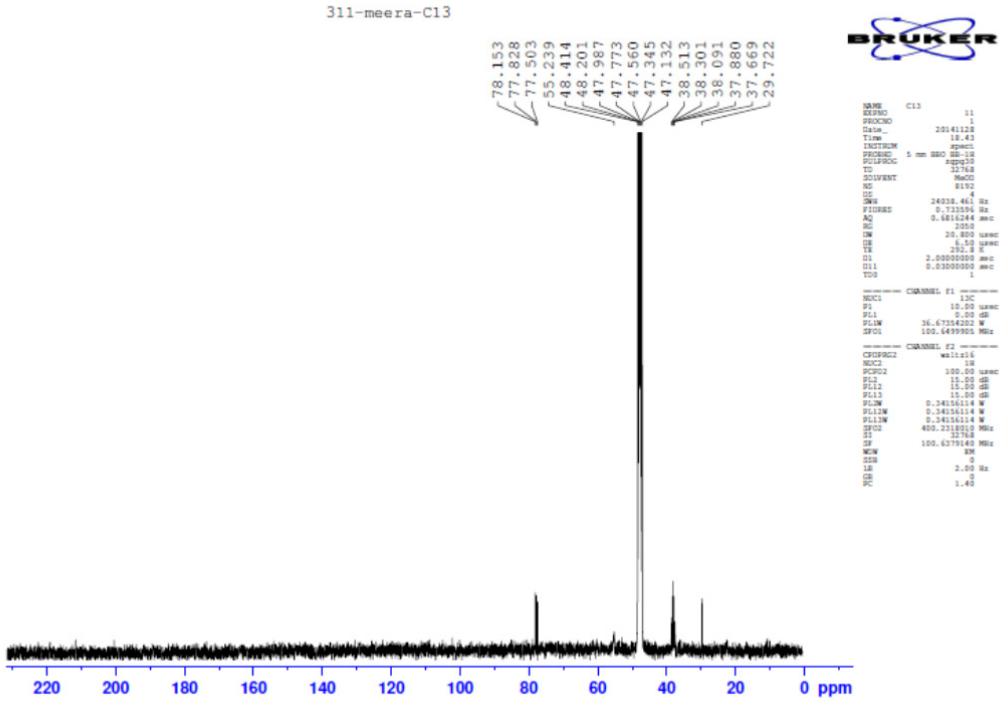
TABLE NO.9

S.No	SIGNALS (δ) VALUES ppm	GROUPS ASSIGNED
1	0.979	Due to CH_3 protons
2	1.2	Due to CH_3 protons
3	1.96	Due to CH_2 attached to C =C
4	2.181	Due to CH_2 proton attached near to CH_2-C=O

IR SPECTRUM OF COMPOUND CM-II



NMR SPECTRUM OF COMPOUND CM -II



CHAPTER IX

PHARMACOLOGICAL SCREENING

PHARMACOLOGICAL SCREENING

ANTI CANCER ACTIVITY OF ETHANOLIC EXTRACT OF *CRATAVEVA MAGNA* LOUR DC AGAINST DALTON'S ASCITIC LYMPHOMA IN MICE

Experimental Design

Selection Grouping and Acclimatization of Laboratory Animal [48]

Male Swiss albino mice (20-25 gm) were produced from animal experimental laboratory, and used throughout the study. They were housed in micro nylon boxes in a control environment (temp 25±2°C) and 12 hrs dark /light cycle with standard laboratory diet and water *ad libitum*. The study was conducted after obtaining institutional animal ethical committee clearance. As per the standard practice, the mice were segregated based on their gender and quarantined for 15 days before the commencement of the experiment. They were fed on healthy diet and maintained in hygienic environment in our animal house.

Technique for Inducing Tumor

Various technique for induction of cancer in animals, viz, chemically induced (using DMBA/croton oil, etc) [49] virus induced, cell line induced (sarcoma – 180, ULCA fibro sarcoma and Jensen sarcoma, mouse lung fibroblast cells L-929, Dalton's Ascites Lymphoma (DAL), Ehrlich Ascites Carcinoma (EAC)[50-52] methods have been used in experimental studies of anticancer activity.

In the present study, cell lines induced cancer in mice was used to evaluate the anticancer activity of ethanolic extract of *Cratavea magna* lour DC.

EVALUATION OF ANTICANCER ACTIVITY

Induction of cancer using DAL cells

Dalton's Ascites Lymphoma (DAL) cells were supplied by Amala cancer research center, Trissur, Kerala, India. The cells maintained in vivo in Swiss albino mice by intraperitoneal transplantation. While transforming the tumor cells to the grouped animal the DAL cells were aspirated from peritoneal cavity of the mice using saline. The cell counts were done and further dilution were made so that total cell should be 1 x 10^6, this dilution

was given intraperitonealy. Let the tumour grow in the mice for minimum seven days before starting treatments.

Treatment Protocol

Swiss Albino mice were divided in to five group of six each. All the animals in four groups were injected with DAL cells (1 x 10^6 cells per mouse) intraperitoneally, and the remaining one group is normal control group.

Group 1 served as the normal control.

Group 2 served as the tumour control. Group 1 and 2 receives normal diet and Water.

Group 3 served as the positive control, was treated with injection 5- fluorouracil at 20mg/kg body weight, Intra peritoneally.[53]

Group-4 served as treatment control received 200 mg/kg EECM administered through orally.

Group-5 served as treatment control received 400 mg/kg of EECM administered through orally.

Treatment

In this study, drug treatment was given after the 24 hrs of inoculation, once daily for 14 days.

On day 14, after the last dose, all mice from each group were sacrificed by euthanasia. Blood was withdrawn from each mouse by retro orbital puncture bleeding and the following parameters were checked.

1. **Hematological parameters**
    a. WBC count
    b. RBC count
    c. Hb content
    d. Platelet count
    e. Packed cell volume

2. **Serum enzyme and lipid profile**

   a. Total Cholesterol (TC)
   b. Triglycerides (TG)
   c. Aspartate amino Transferase (AST)
   d. Alanine amino Transferase (ALT)
   e. Alkaline Phosphatase (ALP)

3. **Derived parameter**

   a. Body weight
   b. Life span (%)
   c. Cancer Cell Count

EVALUATION OF CLINICAL PARAMETERS

Cancer cell count [54]

The fluid (0.1ml) from the peritoneal cavity of each mouse was withdrawn by sterile syringe and diluted with 0.8 ml of ice cold Normal saline or sterile Phosphate Buffer Solution and 0.1 ml of tryphan blue (0.1 mg/ml) and total numbers of the living cells were counted using heamocytometer.

$$\text{Cell count} = \frac{\text{No of cells dilution}}{\text{Area} \times \text{Thickness of liquid film}}$$

1) **Hematological parameters**

   i) WBC count
   ii) RBC count
   iii) Platelet count
   iv) Hemoglobin
   v) Packed Cell Volume

i) WBC count

The total WBC count was found to be increased in cancer control, when compared with normal and treated tumor-bearing mice.[55]

ii) RBC and Hb

RBC and Hb content decreases with tumor bearing mice when compared with Normal control mice.

iii) Platelets

In Hodgkin lymphoma, increased in platelet count often reported in laboratory finding. Hence, I investigated this parameter in the study. [56]

iv) Packed cell volume

In any case of anemia the packed cell volume is decreases.

SERUM ENZYME AND LIPID PROFILE

The serum was analyzed for the following parameters

(a) Aspartate amino Transferase (AST)
(b) Alanine amino Transferase (ALT)
(c) Alkaline Phosphatase (ALP)
(d) Total Cholesterol (TC)
(e) Triglyceride (TG)

1. TOTAL CHOLESTEROL AND TRIGLYCERIDE (lipid profile)

Abnormal blood lipid profile has been associated with cancer. In Hodgkin lymphoma, high cholesterol level and low triglyceride level has been reported. Hence I investigated this parameter in the study. [57]

LIVER ENZYMES (AST, ALT and ALP)

Abnormal liver function seen in patient with Hodgkin lymphoma, that these liver enzyme levels markedly increase in tumor bearing mice. ALP is an enzyme mainly derived from the liver, bones and in lesser amount from intestines, placenta, kidneys and leukocytes.

An increase in ALP levels in the serum is frequently associated with the variety of disease.[58] ALP comprises a group of enzyme that catalyzes the phosphate esters in an alkaline environment, generating an organic radical and inorganic phosphate.

Markedly elevated serum ALP, hyperalakline-phosphatasemia, is seen predominantly with more specific disorders; including malignant biliary cirrhosis, hepatic lymphoma and sarcoidosis. [59] Hence, I investigated this parameter in this study.

DERIVED PARAMETERS

1. Body weight:

All the mice were weighed, from the beginning to 15th day of the study. Average increase in body weight on the 15th day was determined.

2. Percentage increase in life span (ILS)

% ILS was calculated by the following formulae

Life span of treated group

$$\%ILS = \frac{\text{Life span of treated group}}{\text{Life span of control group}} - 1 \text{ x } 100$$

- All biochemical investigations were done by using COBAS MIRA PLUS-S Auto analyzer from Roche Switzerland.
- Hematological test are carried out in COBAS MICROS OT 18 from Roche.
- Newly added Hi-Tech instruments MAX MAT used for an auto analyzer for all biochemistry investigations in blood sample.

Table No. 1

Effect of EECM on Haematological Parameters

Treatment	Total WBC Cells /mlx10^3	RBC Count Mill/cumm	Hb Gm/dl	PCV %	Platelets Lakhs/cumm
G_1	13.85 ±1.80	5.60±0.86	13.65 ±1.30	16.40±2.45	5.46±0.90
G_2	15.65 ±2.60^{a**}	4.48±0.20^{a**}	8.36 ±0.92^{a**}	32.40±3.25^{a**}	3.75±0.62^{a**}
G_3	12.60 ±1.75^{b**}	5.20±0.78^{b**}	12.30±1.45^{b**}	18.40±1.50^{b**}	4.80±0.96^{b**}
G_4	11.42 ±1.90^{b**}	5.45±0.58^{b**}	11.40±1.32^{b**}	22.40±1.70^{b**}	4.15 ±0.80^{b**}
G_5	13.40±1.75^{b**}	5.25±0.50^{b**}	11.5±1.05^{b**}	23.26±1.85^{b**}	4.30±0.92^{b**}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 – Treatment control 200mg/kg EECM, G5 – Treatment control 400 mg/kg EECM

All values are expressed as mean ± SEM for 6 animals in each group.

**a – Values are significantly different from control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

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Table No.2

Effect of EECM on serum Enzymes and lipid proteins

Treatment	Cholesterol (mg/dl)	TGL (mg /dl)	AST (U/L)	ALT (U/L)	ALP (U/L)
G_1	125.30±3.70	138.60±2.55	43.65 ±1.35	34.50 ±1.55	121.40 ±2.45
G_2	154.90±4.65^{a**}	223.40±4.85^{a**}	89.6±2.70^{a**}	64.45±2.70^{a**}	230.45±4.36^{a**}
G_3	132.50±3.90^{b**}	168.65±2.45^{b**}	56.48 ±1.85^{b**}	45.50±1.85^{b**}	169.45±2.55^{b**}
G_4	134.55±4.15^{b**}	172.30±2.85^{b**}	64.50±1.95^{b**}	47.36 ±1.98^{b**}	188.65±2.70^{b**}
G_5	134.50±3.25^{b**}	171.60±2.60^{b**}	62.65 ±2.35^{b**}	46.30±1.75^{b**}	188.42±2.35^{b**}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 – Treatment control 200 mg/kg EECM, G5 – Treatment control 400 mg/kg EECM)

All values are expressed as mean ± SEM for 6 animals in each group.

**a – Values are significantly different from control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

Table No.3

Effect of EECM on the life span, body weight and Cancer cell count of tumor induced mice

Treatment	Number of animals	% ILS Life span	Increase in Body weight grams	Cancer cell count ml X 10^6
G_1	6	>>31 days	1.70±0.62	-
G_2	6	49%	6.80±0.96^{a**}	3.70±0.40^{a**}
G_3	6	92%	4.85±0.70^{b**}	2.59±0.22^{b**}
G_4	6	88%	5.55±0.88^{b**}	2.80±0.30^{b**}
G_5	6	87%	5.70±0.86^{b**}	2.78±0.25^{b**}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 – Treatment control 200mg/kg EECM, G5 – Treatment control 400 mg/kg EECM

All values are expressed as mean ± SEM for 6 animals in each group.

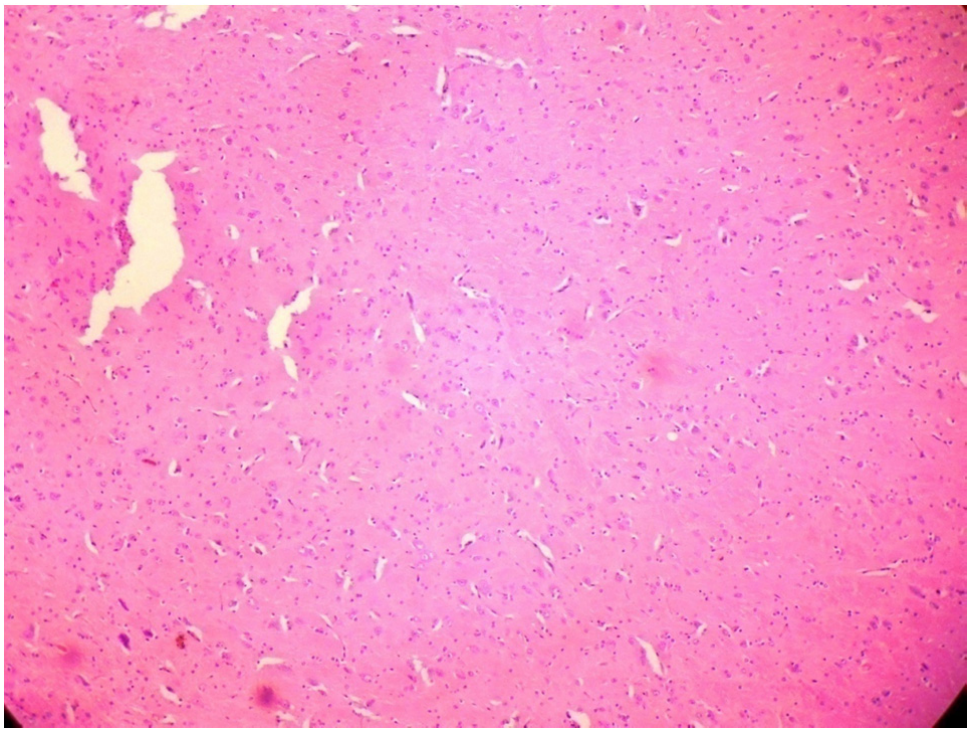
**a – Values are significantly different from control (G_1) at $P < 0.01$

**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

HISTOPATHOLOGICAL RESULTS

Figure No. 1

Normal Control Group

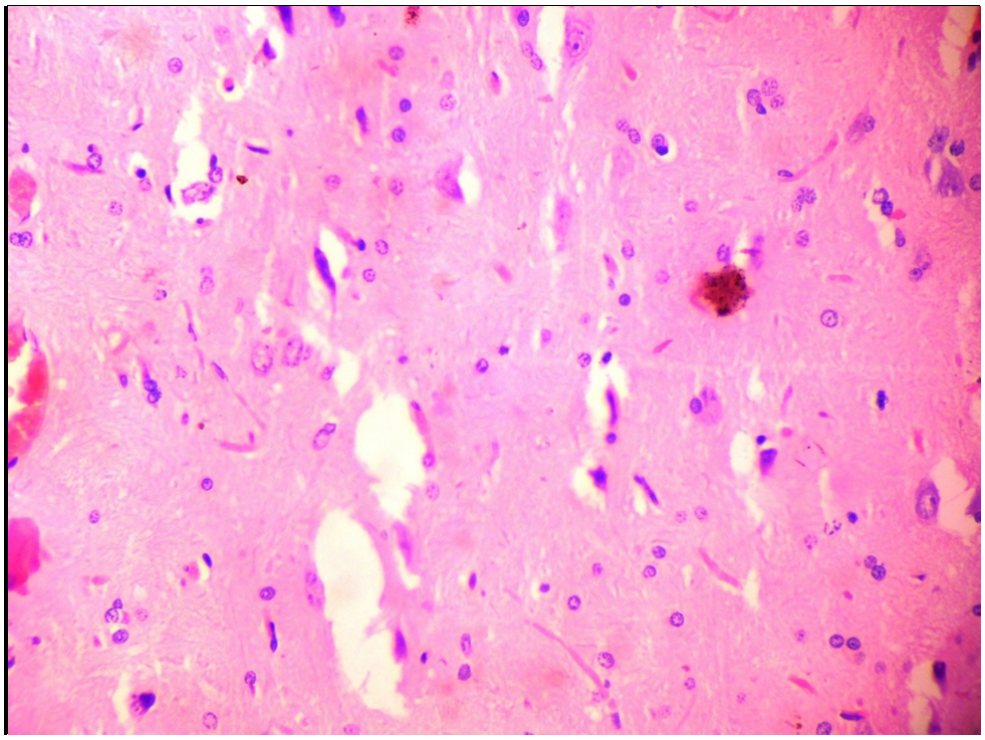


Section shows Structure of Liver with sheets of Hepatocytes Separated by

Sinusoids Cartial Vein & Portal tract appear normal

Figure No. 2

Toxic control group (Tumour Control)

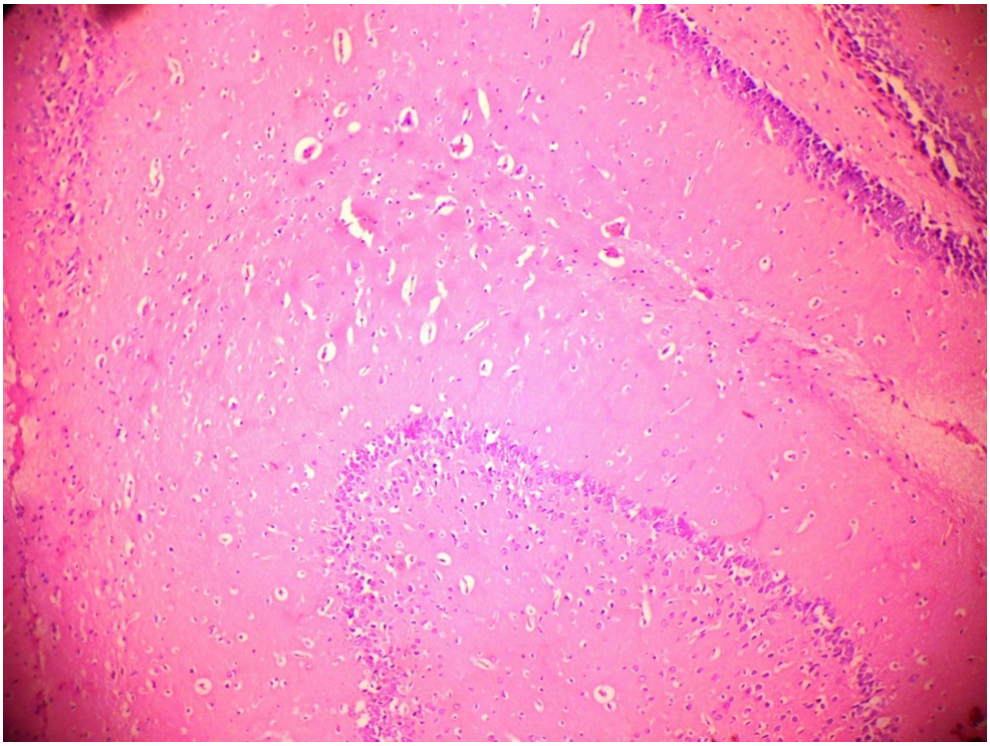


Sections shows structure of liver with cords of hepatocytes and

small area of lymphmatous cells

Figure No.3

(Standard Control)



Positive control, was treated with injection fluorouracil at

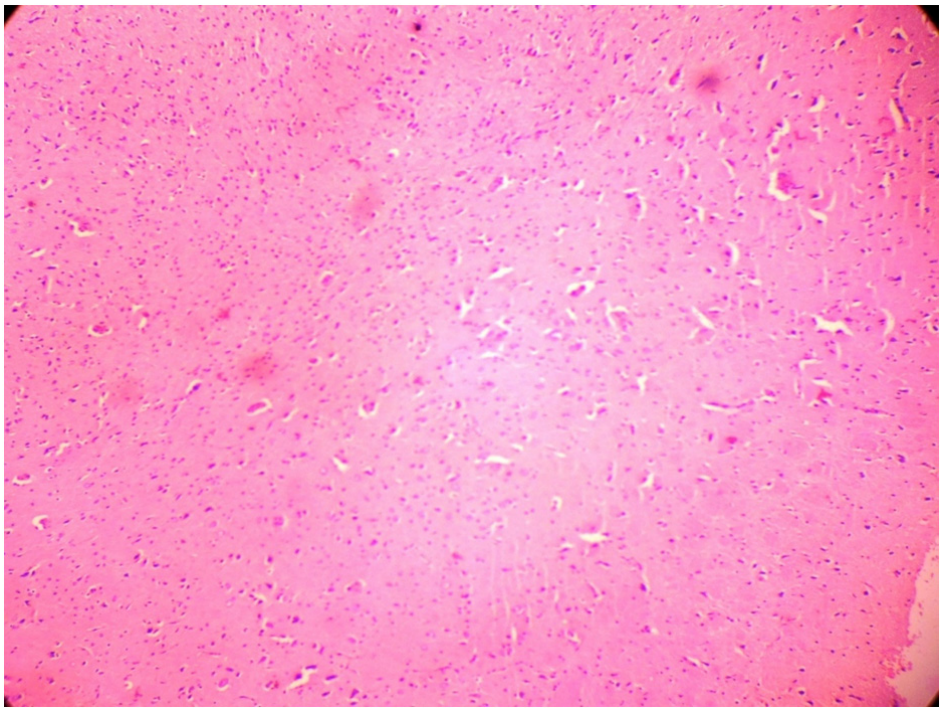
20 mg/kg body weight, Intra peritoneally

Structure of liver with cords of hepatocytes without tumor

Figure No .4

(Treatment Control)

Treatment control received 200 mg/kg of EECM administered through orally



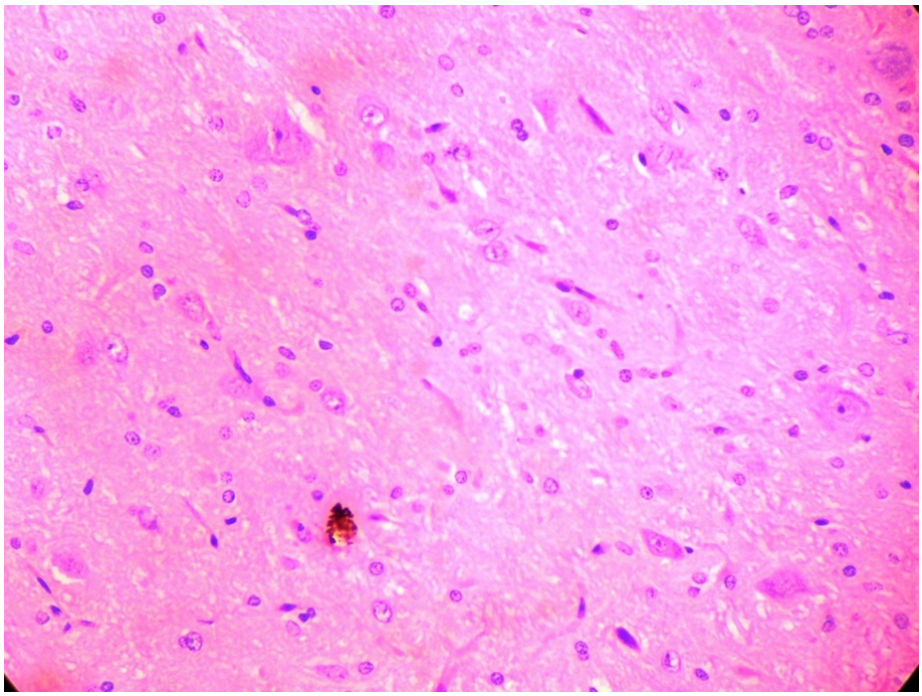
Section show structure of liver with cords of hepatocytes.

No tumor tissue seen

Figure No. 5

(**Treatment Control**)

Treatment control received 400 mg/kg of EECM administered through orally



Sections show liver parenchyma with cords of hepatocytes.

No tumor tissue seen

CHAPTER X

RESULTS & DISCUSSION

RESULTS & DISCUSSION

For the sole reason that *Crataeva magna* Lour DC possess various phytoconstituents and pharmacological properties, we resolved to work on root bark of *Crataeva magna* Lour DC, which are widely used in medicines of natural products.

The root bark was shade-dried and ground to coarse powder and extracted in series, using Petroleum ether AR, Chloroform AR and Ethanol AR in an increasing order of polarity

Phytochemical Screening of *Crataeva magna* Lour DC was undertaken to study, isolate and characterize the chemical constituents and the Pharmacological activities.

During our investigation, the Pet ether extract, chloroform extract, Ethanol extract have revealed the presence of Glycosides, Alkaloids, Carbohydrates, Phytosterols, Saponins, Fixed oils, Fats, Flavonoids and Coumarins and these constituents were isolated extraction with Chloroform AR.

The Ethanol extract was subjected to Column Chromatography on silica (100-200) mesh. During our investigation about two Compounds were isolated.

CM-I & CM-II

These compounds eluted with solvents of increasing polarity like Petroleum ether, Chloroform, Ethyl acetate and methanol. In the course of isolation procedure, the above named 2 compounds were further processed and characterised.

The compound CM-I is Dark green semisolid in appearance. In TLC, it showed a single spot using Benzene: Chloroform (8:2) have R_f value 0.42 and the melting point is 142°C. The IR & ^{1}H NMR Spectral data showed the characteristic feature of Alkaloids and which was also confirmed by chemical test.

The compound CM-II is green viscous in appearance. In TLC, it showed a single spot, using chloroform: Ethyl Acetate (7:3) have R_f value 0.71 and the melting point is 138°C. The IR & ^{1}H NMR Spectral data showed the characteristic feature of Glycosides and which was also confirmed by chemical test.

Effect on Tumour Growth

In the DAL tumour control group, the average life span of animal was found to be 49% where as EECM at a dose of 200 mg and 400 mg/kg body weight increase the life span to 88%, and 87% respectively. These values were significant. However the average life span of 5- FU treatment was found to be 92%, indicating its potent antitumor nature. The antitumor nature of *Cratavea magna* Lour DC were evidenced by the significant reduction in percent increase in body weight of animal treated with *Cratavea magna* Lour DC when compared to DAL tumour bearing mice.

It was also supported by the significant reduction in packed cell volume and viable Tumour cell count in EECM at a dose of 200mg and 400/kg body weight treatments when compared to the DAL tumour control. (Table No 2 & 3).

Effect on Hematological Parameters

As shown in (Table No.1) RBC, Hgb, Platelets were decreased and WBC count was significantly increased in the DAL control group compared to the normal control group. Treatment with *Cratavea magna* Lour DC significantly increases the Hb content, RBC, Platelets and significantly decreased the WBC count to about normal level. All these results suggest the anticancer nature of the *Cratavea magna* Lour DC. However, the standard 5-FU at the dose of 20 mg/kg body weight produced better result in all these parameters.

Effect on Biochemical Parameters

The inoculation of DAL cells caused significantly increase in the level of Total Cholesterol, Aspartate amino Transferase, Alanine amino Transferase, Alkaline Phosphatase in the tumor control animals (G_2), when compared to the normal group. The treatment with *Cratavea magna* Lour DC weight reversed these changes towards the normal level. (Table No. 2) All the value was found to be significant. The treatment with standard 5- FU also gave similar results.

The alternative system of medicines like Ayurvedic, Siddha, Unani and other tribal folklore medicines have significantly contributed to the health care of the population of India. Today these systems are not only complementary but also competitive in the treatment of various diseases. Plants have served as a good source of antitumor agents. Several studies have been conducted an herbs under a multitude of ethanol botanical grounds. A large number of plants possessing anticancer properties have been documented.

Plants of ethanolic extract of *Crataeva magna* Lour DC was traditionally used in the treatment of tumors. The present investigation was carried out to evaluate the antitumor activity of ethanolic extract of *Crataeva magna* Lour DC in DAL tumor bearing mice. The ethanolic extract of *Crataeva magna* Lour DC treated animals at the doses of 200&400 mg/kg significantly inhibited the tumor volume, packed cell volume, tumor (viable) cell count and brought back the haematological parameters to more or less normal levels.

In DAL tumor bearing, a regular rapid increase in ascitic tumor volume was observed. Ascitic fluid is the direct nutritional source for tumor cells and a rapid increase in ascitic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells. Treatment with *Cratavea magna* Lour DC inhibited the tumor volume, viable tumor cell count and increased the life span of the tumor bearing mice. The reliable criteria for judging the value of any anticancer drug are the prolongation of the lifespan of animals. It may be concluded that by decreasing the nutritional fluid volume and arresting the tumor growth increases the life span of DAL bearing mice. Thus *Cratavea magna* Lour DC at a dose of 200 mg and 400 mg/kg body weight have antitumor activity against DAL bearing mice.

Usually, in cancer chemotherapy the major problems that are being encountered are of myelo suppression and anemia. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions. Treatment with Ethanolic extract of *Cratavea magna* Lour DC brought back the hemoglobin (Hb) content, RBC and WBC count more or less to normal levels significantly. This clearly indicates Ethanolic extract of *Cratavea magna* Lour DC possess protective action on the haemopoietic system.

It was reported that the presence of tumour in the human body or in the experimental animals is known to affect may function of the liver. The significantly elevated level of total cholesterol, TG, AST, ALT and ALP in serum of tumour inoculated animal indicated liver

damage and loss of functional integrity of cell membrane. The significant reversal of these changes towards the normal by Ethanolic extract of *Cratavea magna* Lour DC treatments.

In the present study, the biochemical examination of DAL inoculated animals showed marked changes indicating the toxic effect of the tumour. The normalization of these effects observed in the serum treated Ethanolic extract of *Cratavea magna* Lour DC weight supported the potent antitumor and hepatoprotective effect of the Ethanolic extract of *Cratavea magna* Lour DC.

CHAPTER XI

CONCLUSION

CONCLUSION

As is evident from our experiments that Phytoconstituents present in these extracts are too many, it is decided to further up our exercise to isolate and characterize each and individual compound. During our course of Pharmacological activates both Ethanolic and Aqueous extracts, when compared to standard showed almost equipotent significance in respect of the protocol studies.

As on date, special care and attention has been converged on the Phytochemical and Degradation studies and the role and importance of natural products of medicinal interest. Nowadays the research on main source of novel-lead substances for drug design and modeling. The natural and synthetic Phytoconstituents constitute a prime and vital role in Pharmacological, Clinical and Cytotoxic studies leading to an important impact in preparing natural-product-based libraries for Combinatorial Chemistry.

Eventually, a sociology of this extent, provide a constructive basis upon which verified and detailed investigations of various parameters on therapeutic efficacy of natural products leading to excellent use of indigenous medicine is achieved.

In the conclusion, *Crataeva magna* Lour DC was selected for the present study based on its traditional claim. The Ethanolic extract of *Crataeva magna* Lour DC possess significant anti cancer activity against DAL and EAC induced in mice. This herb was effective in inhibiting the tumour growth in ascitic and solid tumour models. The biochemical and histopathological studies also supported its antitumor properties. However, further researches are required to isolate and identify the active constituents for anti cancer agents from the plant as well as elucidating their possible mechanism of action.

CHAPTER XII

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